cancer treatment reports

Proceedings of the 4th Conference on Immune Modulation and Control of Neoplasia by Adjuvant Therapy

Volume 62
Number 11
November 1978
Pages 1611-1998

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service National Institutes of Health

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through January 31, 1980.

Proceedings of the 4th Conference on Immune Modulation and Control of Neoplasia by Adjuvant Therapy

Division of Cancer Cause and Prevention
National Cancer Institute

Editor: Michael A. Chirigos

March 29–31, 1978
Bethesda, Maryland

Introduction

An impressive literature has developed concerning mechanisms by which the immune response can be stimulated. The ability to modulate host immunity is now a reality and the rapidly developing fields of immunotherapy and immunopharmacology are becoming important areas of cancer therapy. Since the immunologic surveillance of the cancer patient is suppressed both by the disease itself and by the cytoreductive therapy required to control it, the early return of host immune function is of major importance. The availability of several chemical and biologic agents capable of reconstituting and/or stimulating the cellular components of the immune response provides the cancer therapist with greater flexibility in developing different combined-modality treatment regimens.

This volume contains the most recent information concerning a few of the more promising immunoadjuvants. Levamisole has been in clinical trial as an adjuvant to chemotherapy and/or surgery for the treatment of solid tumors and leukemias. The responses achieved indicate that it exerts a beneficial effect in reconstituting T-cell depression. For the first time, thymosin has been demonstrated to be an effective adjuvant in the treatment of human carcinomas. Agents such as interferon and glucan appear to stimulate an immune response.

Several agents, through experimental testing in human and animal test systems, possess potential as immunoadjuvants. BM 12.513, isoprinosine, thiobendazole, and maleic anhydride-vinyl ether copolymer are but a few chemically defined chemicals along with *Brucella abortus* extract which exert an immunostimulating effect on one or more of the cellular components of the immune system.

As the chemotherapist has developed combined drug therapy for more effective cancer treatment, so can the immunotherapist and immunopharmacologist develop chemical and biologic agents which can be used in concert with chemotherapy, surgery, or irradiation to reconstitute and stimulate host immune function for the benefit of the cancer patient.

I would like to thank my associates and the many participants for their help and encouragement in making this Conference a very informative and successful one.

Michael A. Chirigos

National Cancer Institute
Bethesda, Md

Pharmacologic Factors and Manipulation of Immunity Systemic Adjuvants in Cancer Therapy

Georges Mathé,* Irène Florentin, Lennart Olsson, Martine Bruley-Rosset, Jan Schulz, Nicole Kiger, Simone Orbach-Arbouys, Léon Schwarzenberg, Pierre Pouillart, and Francoise de Vassal [1]

SUMMARY

Because of the experimental and clinical studies which have been extensively conducted with bacillus Calmette-Guérin (BCG) as a systemic adjuvant in cancer immunotherapy, we have analyzed the main factors and conditions which determine its beneficial action and have underlined some of these (eg, the dose factor which controls the amplification of suppressor cells which is probably responsible for failures and even the possible tumor-enhancing effect of immunotherapy). Knowing those factors and conditions, we have been able to establish a systematic immunopharmacologic study of systemic immunity adjuvants, which has resulted in the discovery of agents whose actions are more rapid than that of BCG on one or a few populations of cells involved in immunity and which, unlike BCG, do not induce suppressor cell amplification. This amplification may explain the difference in the results obtained with this mycobacterium in various clinical immunotherapy trials in which it was applied differently. It is proposed to combine these mono- or pauci-functional adjuvants in order to try to obtain all of the beneficial effects of BCG without the amplification of suppressor cells.

[Cancer Treat Rep 62:1613–1621, 1978]

Sixteen years ago we first attempted to use immunotherapy as a means to eradicate minimal residual disease left by other treatments. This minimal residual disease, which is responsible for local or metastatic relapses, and therefore death, is very frequent after the local treatment of most tumors. In the case of breast carcinoma, the cure rate is 10% at the 20th year according to Brinkley and Haybrittle (1).

Postsurgical systematic chemotherapy, so-called adjuvant chemotherapy, has given great hope of being able to eradicate this residual disease. While the results are very encouraging in premenopausal patients with breast carcinoma, they are not significant in postmenopausal patients (2,3). In osteosarcoma, while the benefit of adjuvant chemotherapy appeared enormous at the 18th month, it was significantly reduced at the fifth year (4). In the case of carcinoma of the bronchus, postsurgical chemotherapy has not been shown to be beneficial in any trial (5).

In the future, chemotherapy will certainly be made more efficient with the use of drug combinations and improvement in their application. However, there are two stumbling blocks which are particularly difficult to ignore: (*a*) chemotherapy obeys first-order kinetics (6–8), and (*b*) in tumor cell populations there is a certain proportion of cells resistant to the drug(s) (of the order of 10^{-6}) which increases under chemotherapy (10^{-4}) (9). Hence, chemotherapy appears not to be able to kill "the last cell" (10), even if the dose of the drug is maximally increased (11).

It has been estimated by Salmon (12) from data on kinetics and survival that postsurgical chemotherapy must reduce the tumor cell number to ten in order to save 100% of a population of breast carcinoma patients. It can then be supposed that the ten cells that are left are killed by natural immune resistance. This hypothesis fits very well

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with the mean inductive number of L1210 leukemia cells in mice (precisely ten in normal animals which drops to one in immunologically suppressed animals [13,14]).

Because this mean inductive number can be increased to 10^5 by prophylactic immune manipulation we decided to use such a manipulation for the treatment of an established leukemia. Our first experiment showed that such an immunomanipulation called "immunotherapy" was able to delay or reduce the mortality, provided the animals were not carrying more than the key number of 10^5 neoplastic cells (15).

Bacillus Calmette-Guérin (BCG) Immunopharmacology and Immunotherapy

In this experiment living fresh BCG was used as immunity adjuvant at a dose of 1 mg/mouse iv (15); later we observed that it could be efficient when applied via scarifications or with a heaf gun, but not by other routes (16). The reason for this restriction in the routes of administration of BCG was demonstrated by an experiment which we conducted later which showed a correlation between the antitumor effect and the establishment of septicemia (17). This is why we correlate in man the dose of BCG given via scarifications to the delayed hypersensitivity reactivity of the patients. We administer larger doses of BCG to the positive patients than to the negative ones (since more bacterias are killed by the positive subjects), and we consider as a necessary proof of good application the appearance of fever a few hours after BCG administration.

The first clinical attempt to use immunotherapy with BCG as an adjuvant to control the minimal residual disease was a pilot trial which we conducted on acute lymphoid leukemia (ALL) in 1962 (18). Seven of 20 patients who received immunotherapy are still in remission without having relapsed versus 0 of ten controls left without treatment after stopping chemotherapy (19). Today the chemotherapy of this neoplasia has made progress (20–22) and the only ethical trial which can be conducted consists of comparing chemotherapy and immunotherapy. This is what was done by Mathé et al (23) and by the European Organization for Research on Treatment of Cancer Haemopathy Working Party (24) which compared short-term chemotherapy followed by immunotherapy and long-term chemotherapy followed by the same immunotherapy. In both trials, immunotherapy has been as efficient as the best chemotherapy for the periods during which they were compared (between the 9th and the 25th months in our trial).

Other trials using BCG following radiotherapy or chemotherapy in lymphosarcoma (25,26) and after surgery in bronchogenic carcinoma (27) and in melanoma (28) have benefitted the patients submitted to this BCG adjuvant therapy compared to randomized controls. However, even with trial results favoring BCG, there are others which were negative (29). Since the trials differ in the preparations of BCG used, in their modalities of administration, and in the conditions of the disease, it is necessary to search for the factors which may determine the action of BCG.

The first factor shown in mice is the adjunction of specific immunotherapy to the nonspecific immunotherapy done with BCG (30).

The second factor is the preparation of BCG used. At the same optimal dose (1 mg/mouse) we showed that the fresh Pasteur preparation of living organisms is much more efficient than all other preparations in the different tests of our experimental screening for immunity adjuvants usable in cancer chemotherapy (31). This has been confirmed by R. W. Baldwin (personal communication).

The third factor which was already considered above is the route of administration. In mice we showed that regional administration may be efficient when combined with tumorectomy (32) or may add to the effect of systemic iv administration (33). McKneally et al (34) obtained a very significant benefit in patients with stage I bronchogenic carcinoma by injecting BCG intrapleurally at the time of surgery, which represents simultaneously a regional and a systemic immunotherapy. These authors obtained no effect in stage II patients, illustrating with this trial the role of the stage of the disease. Similarly, Economides et al (33) obtained a favorable effect in B16 melanoma with preoperative regional BCG only if the draining lymph nodes were removed.

We have shown that the antibody response to sheep red blood cells was maximally enhanced in mice treated with a dose of 1 mg of BCG, whereas it was depressed after 5- and 10-mg doses (35). The comparison of the effects of different doses of BCG in leukemic mice (36) showed that weekly 1-mg injections cured a certain percentage of the animals whereas weekly 3-mg injections accelerated tumor growth. Induction of suppressor cells may explain these results.

Florentin et al (37) and Orbach-Arbouys and Poupon (38) effectively demonstrated the presence of suppressor cells, identified as macrophages and T cells in the spleens of mice given "high" doses of BCG.

We are presently studying the time factor in the modality of BCG administration. The preliminary results show that five successive injections of 0.2

mg are generally more efficient in potentiating antibody responses than one injection of 0.2 mg, but no more efficient than a single injection of 1 mg. Similarly, five injections of 1 mg are as active as one injection of 5 mg in the induction of T cells and macrophage suppressor cells.[2] There may be an exception for K-cell amplification; five injections of 0.25 mg are not only more efficient than one injection of 0.25 mg, but are more efficient than one injection of 1.25 mg (39).

All of these factors should be known to clinicians since they may guide them to an optimal application of BCG therapy and may help them to avoid any deleterious effects which may result from the action of suppressor cells.

BCG Immunotherapy-Chemotherapy Combination

This possible action of suppressor cells might be one of the most important stumbling blocks of active immunotherapy. Several researchers are trying to eliminate them with cytostatic agents. Polak and Turk (40) succeeded with cyclophosphamide (CPM) and in our laboratory Orbach-Arbouys et al (41) succeeded with methotrexate. This notion strongly supports the purpose of combining, for example by interspersion, chemotherapy and BCG immunotherapy. There are other reasons for such a combination.

Observing the tumor volume curve during active immunotherapy in sc grafted L1210 leukemia, we obtained three kinds of results: (*a*) a decrease of the tumor volume, (*b*) a progressive increase of tumor load until death, and (*c*) a plateau in the tumor volume which is followed by progressive growth until death, suggesting that in this latter case, after the initial antitumor effect, immunoresistance has developed (13,14). This observation suggests the possible usefulness of interspersing immunotherapy and chemotherapy when the tumor volume curve has attained the plateau, in order to reduce the number of tumor cells to a level accessible to immunotherapy ($\leq 10^5$ cells).

In a study conducted on the effect of immunotherapy on the different phases of the tumor cell cycle, we showed (42) that BCG only works on cells in G_0 and/or G_1 phases. These data further support the notion of interspersing immunotherapy and chemotherapy since the latter mainly works on cells in the other phases of the cell cycle (43,44).

Another reason in favor of interspersing active BCG immunotherapy and chemotherapy is the acceleration by BCG of blood cell restoration after chemotherapy (45). This suggests that this adjuvant acts on stem cells. We have observed (46,47), using the techniques of colony-forming units in the spleen and in agar culture, that BCG increases the number of both types of stem cells in S phase.

The significant increase of null cells in the blood of our ALL patients submitted to active immunotherapy compared to those submitted to chemotherapy and to normal controls (48) is also in favor of the action of BCG on cells which are not fully differentiated, and this may be an important mechanism of action of immunotherapy. We were therefore encouraged to conduct immunotherapy-chemotherapy interspersion studies. In our first experiments, we interspersed CPM and BCG in the treatment of L1210 leukemia in which we had previously seen that the sequence of CPM followed by BCG is much more efficient than CPM alone (49). We were unpleasantly surprised, however, to observe that two or three interspersion sequences of these two agents were no more efficient than CPM alone and were much less efficient than the single sequence of one CPM and one BCG injection (50). The same result was observed for the solid Lewis lung tumor (50).

Since the single sequence of CPM followed by BCG is more effective than CPM alone (49), we wondered if the reverse sequence of BCG followed by CPM was less effective; this indeed was the case (49). We had shown that the CPM effect is much poorer in immunodepressed animals (51,52) and we were curious to know whether BCG followed by CPM induced immunodepression since BCG pushes the lymphocytes into the cycle, rendering them more sensitive to the lymphostatic action of CPM, a cycle-dependent agent. An experiment on allogeneic skin grafting (54) demonstrated the validity of this hypothesis: the sequence of BCG followed by CPM greatly prolonged allograft survival compared to CPM alone.

We have investigated whether this phenomenon was true for all chemotherapy agents, or only for those that are known to be immunosuppressive (54,55). Hence, we performed a similar experiment using RFCNU [(chloro-2-ethyl-1-ribofuranosylisopropylidene-2′,3′-paranitrobenzoate-5′)-3-nitrosourea], a nitrosourea derivative which, among those available in practice, was shown to be strongly oncostatic in our laboratory and had no immunosuppressive action at the minimal oncostatic dose (56). The sequence of BCG followed by RFCNU is more efficient in limiting tumor growth than BCG or RFCNU alone (57). Moreover, we have observed that this derivative may exert some immunostimulatory ef-

[2] Florentin I, Bruley-Rosset M, Kiger N, et al. Comparisons of the effects of acute versus chronic administration of BCG on immune responses in young adult and age-immunodepressed mice. Manuscript in preparation, 1978.

fects. It potentiates delayed-type hypersensitivity reaction and induces macrophage activation (57).

Thus, we may conclude that the success of interspersion of immunotherapy and chemotherapy depends at least on one factor: the effect of the chemotherapy on immunity. Nonimmunosuppressive cytostatics must be chosen according to the available data. This effect on immunity does not eliminate the possible role of other factors, such as time, dose, the phase dependency of the cytostatics, or the adjuvant used for immunotherapy (13,14). Clinicians have not waited until experimental models were able to give a rational basis for chemotherapy-immunotherapy interspersion.

Today the literature is rich in results which are far from being in agreement with each other, even when they concern the same disease. Acute myeloid leukemia was the first neoplasia in which BCG (with or without neoplastic cells) interspersed with chemotherapy was shown to be more effective by most trials than chemotherapy alone by most trials. The first such trial was conducted by Powles et al (58); the last trial was published by Udem et al (59).

The recent preliminary results of two trials on advanced cancer of the ovary are especially encouraging. They showed the superiority of chemotherapy-BCG immunotherapy interspersion over the same chemotherapy alone (60,61).

Chemotherapy-BCG immunotherapy interspersion has also been tried in advanced diseases, with favorable results obtained by BCG in melanoma, breast cancer, and neuroblastoma, and negative results obtained in gastrointestinal tract tumors (29). Under the above conditions, immunotherapy has been interspersed with chemotherapy and, as we have shown in mice, a given chemotherapy is much less efficient in immunodepressed animals than in normal ones (52). Hence, another mechanism of action for this combination may be the amplification of the efficacy of chemotherapy by immunotherapy.

This notion led us to submit the skin-test negative bronchogenic carcinoma patients to BCG treatments preceding chemotherapy. We have seen that the survival of the patients whose delayed hypersensitivity responses were restored by BCG was as good as the survival in those who had positive skin tests at the time of diagnosis (62,63).

Hence, the combinations of chemotherapy and BCG immunotherapy are potentially promising and need more experimental and clinical investigations, especially as far as their inter-relationship is concerned. This may vary not only with different kinds of immunotherapy, ie, the type of the preparations and the doses of BCG, but also according to the different cytostatics. The latter should not be regarded as generally immunosuppressive, since they may trigger an immune rebound after having induced immunosuppression (64), or they may activate macrophages themselves (57).

All of these studies were conducted with BCG immunotherapy. The numerous conditions of action of this material as well as the medieval aspect of a therapy consisting of inducing an infection may place and keep immunotherapy below its real potential, although we now know several reasons for its beneficial effect or the lack of it. Furthermore, we may even have an explanation for its deleterious effect, this being possibly due to the amplification of suppressor cells by a dose higher than the optimal one. This is why many researchers are tempted to replace BCG with other immunity adjuvants.

The first attempts were made with BCG extracts. We have observed that if most of the extracts, including the hydrosoluble ones, are efficient in the antibody production and in macrophage activation tests, only the methanol-extracted residue fraction (MER) is active in tumor-growth inhibition (65). However, the clinical tolerance of MER is poorer than that of BCG. Pure molecules such as muramyl dipeptides and their derivatives have now been extracted and even synthesized. Some have been tested in local immunotherapy (66) but have not yet been tested in systemic active immunotherapy (67).

Other Micro-organisms

Killed organisms other than BCG have been studied. The factors determining their action have not yet been analyzed as those described above for BCG.

Corynebacterium parvum has been used extensively by many investigators (68,69), and we have frequently seen a beneficial effect in experimental tumors (70). The depressing effect on T-cell immunity reported by Scott (71) must be considered within the framework of possible amplification of suppressor cells by high doses of some adjuvants as we showed for BCG. The results such as those obtained in a trial on melanoma, in which the survival of the *C. parvum* branch deteriorated compared to that of controls (72), could probably be avoided.

We are interested in the action of *Brucella* (73), which in our screening[3] again illustrates the importance of a phenomenon we noted for BCG, namely, that the adjuvant action depends on the strain of

[3] Mathé G, Halle-Pannenko O, Florentin I, et al. New agents submitted to EORTC-ICIG experimental screening for systemic immunity adjuvant applicable to cancer immunotherapy. Manuscript in preparation, 1978.

micro-organism used. Only the B19R strain is efficient, and only at certain doses, while the B19S strain is immunosuppressive and enhances tumor growth.

Finally, we have recently obtained promising results with *Pseudomonas aeruginosa*, both in our study in mice (74,75) and in a clinical study on immunorestoration of delayed hypersensitivity in immunodepressed patients (76). We do not yet know the significance of this clinical test for immunorestoration of delayed hypersensitivity-negative cancer patients in whom the negativity is not due to radiotherapy or chemotherapy. It is an easy test for clinical phase II trials of immunity adjuvants and their means of administration as illustrated by the effect of BCG. Immunorestoration is only obtained with small doses of BCG which are inefficient in delayed skin hypersensitivity responders, while large doses are necessary for inducing an amplification of some immune reactions in those patients (77). One can also see that not all agents can induce immunorestoration since we were unable to obtain it in any of the patients treated with poly I:poly C (78).

Chemically Characterized Adjuvants

Levamisole

The above observations and the possible importance of immunorestoration of immunodepressed patients before chemotherapy (62,63) led us to set up an experimental screening on immunorestoration. The most interesting experiment proved to be the study of the effects of agents on aged mice (12–18 months old). We first studied levamisole which was then compared to BCG. While repeated administration of BCG depresses T-dependent antibody production and induces suppressor cells, repeated administration of levamisole restores this antibody production without inducing suppressor cells (79).

This immunorestoring effect of levamisole which does not induce suppressor cells may explain five remarkable clinical results obtained in randomized trials concerning ALL (80), cancer of the bronchus (81), breast cancer (82,83), melanoma (84), and head and neck carcinoma (85).

Thymosin

Our work on immunorestoration with levamisole led us to study thymosin, comparing its effect with BCG in multiple-dose treatment. The main conclusions from this work are as follows: (*a*) thymosin activates macrophages but to a lesser degree than BCG; (*b*) it behaves as levamisole after a certain length of administration, restoring T-dependent antibody production against sheep red blood cells; and (*c*) unlike BCG, it does not induce suppressor cells (86).

The recent clinical result obtained by Chretien (87) in small cell carcinoma of the bronchus with the combination of thymosin and chemotherapy is very encouraging in this regard since his results are particularly significant in the patients who were T-cell immunodepressed.

BM 12531

We are currently analyzing the functions of many chemically well-characterized agents for which there are data suggesting that they exert adjuvant effects, whether they are obtained by extraction, such as bestatine (88), or synthesized, such as 2-[2-cyonazimidinyl-(1)]-2-[2-carbomylyzindinyl-(1)]propan or BM 12531 (89), and we are comparing their respective mechanisms of action. Table 1 shows the actions of several agents.

Most of these agents, unlike BCG and *C. parvum* which amplify all populations of cells involved in immunity (including suppressor cells which may be a stumbling block for their controllable use), act more rapidly on one or a few populations of cells.

Combination of Adjuvants

Our objective is to obtain, by combining these adjuvants, all of the beneficial effects of BCG without the amplification of suppressor cells, and hence to employ agents which will not present the logistic difficulties of BCG application which is used differently in various centers, possibly explaining the contradiction in the results obtained (29).

Today, we may combine an adjuvant working mainly on macrophages such as glucan (90), an adjuvant working on B cells such as *P. aeruginosa* (75) (which in the form of dead micro-organisms is perfectly tolerated in man [76] and does not induce the numerous side effects of *Pseudomonas* lipopolysaccharide [LPS] [91]), and an adjuvant working on T cells such as levamisole or thymosin in T-cell immunodepressed subjects or BM 12531 in nonimmunodepressed patients. The latter also works on null cells.

Hence, if we look for adjuvants well-tolerated and identically administrable in all centers and countries, we can not afford to abandon totally biologic materials and limit ourselves to pure molecules: (*a*) *P. aeruginosa* is better tolerated than the pure LPS; (*b*) there are still activities that could only be obtained with special administration of BCG such as the amplification of K cells or NK cells; and (*c*) it is by sequentially combining BCG or *C. parvum*

TABLE 1.—Comparison of the effects of several adjuvants on immune responses in mice

Adjuvant	Macrophage activation	Stimulation by mitogens of— T lymphocytes	Stimulation by mitogens of— B lymphocytes	Induction of suppressor cells	No. of cells forming lysis plaques to— T-dependent antigen (TNP-KLH)	No. of cells forming lysis plaques to— T-independent antigen (TNP-POL)	Presence of K cells	Delayed hypersensitivity
BCG (1 mg/mouse)	↑↑	↓↓	↑	T-lymphocyte macrophages	↑↑	↑↑	↑↑	↑
LPS (200 μg/mouse)	↑↑	↓	↓	Macrophages	→	↓	↓	Not tested
P. aeruginosa (0.1 ml [10^9/ml]/mouse)	↑	↓	↑	→	Not tested	Not tested	→	→
Levamisole (75-25 μg/mouse)	→		→	?	Not tested	Not tested	→	→
Poly I:poly U (250 μg/mouse)	↑	↓	→	?	↑	↓	↓↓	Not tested
Poly I:poly C (250 μg/mouse)	↑	↓	↑	?	↑	↓	→	Not tested
Bestatine (100 μg/mouse)	→	→	→		↑ (sheep red blood cells)	Not tested	→	→
BM 12531 (100 μg/mouse)	↑	→	→	↑	↑	→	Not tested	↑

with LPS from *P. aeruginosa* (92) or with the killed form of this micro-organism[4] that one is able to induce tumor necrotizing factor production. This factor may represent a new bridge between immunotherapy and chemotherapy as it is simultaneously an immunity adjuvant (93) and a cell poison. Moreover, when creating our chemotherapy-immunotherapy combinations, we must not forget that certain cytostatics, such as some new nitrosoureas, are able by themselves to activate macrophages (57).

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Immunopotentiation and Tumor Inhibition With Levamisole

Derek Sampson [1,*]

SUMMARY

The immunopotentiating drug, levamisole, was found to augment human lymphocyte responses to vegetable mitogens in vitro. However, the effect was dose-dependent and at high concentrations suppression rather than augmentation of the immune response was observed. In view of the potential widespread use of the drug in the treatment of human cancer, a study was undertaken in rats bearing breast cancer to determine whether a similar dose-response effect would be observed in terms of tumor growth. Tumor inhibition was found to be dose-dependent, and at high doses tumor inhibition did not occur while at lower doses the growth of the cancer was inhibited. The rate of tumor growth correlated well with the responses of splenic lymphocytes to vegetable mitogens. In a later study, it was not possible to show that levamisole had any effect in boosting immune responses of patients undergoing surgery. It is concluded that levamisole is capable of increasing immune responses in man in vitro, and that the drug is able to inhibit the growth of breast cancer in the rat. However, these effects are dose-dependent, and clinical trials of the use of levamisole in patients with cancer should include careful immune monitoring to be sure that augmentation rather than suppression of the immune response is being achieved.

[Cancer Treat Rep 62:1623–1625, 1978]

There are now > 400 reports documenting the fact that levamisole is able to amplify the immune response both in vitro and in vivo, in animals and man, and that the drug is able to inhibit the growth of solid tumors and leukemias. Antiviral activity has also been documented. However, there are many inconsistencies in these reports, some showing effects and others not. Little attention has been paid to the dose-response effects of the drug. The studies reported in this paper were designed to assess the importance of dose, and once it was appreciated that this was important in human in vitro studies, experiments were subsequently designed to test the relevance of dose in an animal tumor model.

MATERIALS AND METHODS

For the human in vitro studies, peripheral blood lymphocytes were obtained by density gradient separation, using Ficoll-sodium metrizoate, and were then incubated with three different mitogens, ie, phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM). During the 3-day incubation, graded doses of levamisole were added to cultures up to a concentration of 1.6 μg/ml. At the end of the incubation, mitogen response was assessed by the addition of tritiated thymidine, and the degree of augmentation was calculated from the ratio of cpm per culture with levamisole compared to the cpm for the culture without the drug. The methods have been described fully elsewhere (1). For the animal studies, breast cancer was induced in Sprague-Dawley rats by the administration of 7,12-dimethylbenz(α)anthracene and divided into groups. Once tumors had reached 0.5 cm in diameter the rats were given levamisole orally in doses of 0, 2, 4, or 8 mg/kg of body weight and tumor size was monitored for 6 months from the start of the study. At this time the animals were killed and lymphocyte suspensions were prepared from the spleen and tested for responses to the three vegetable mitogens PHA, Con A, and PWM. The details of this study have been previously reported (2), and the experimental design is shown in figure 1.

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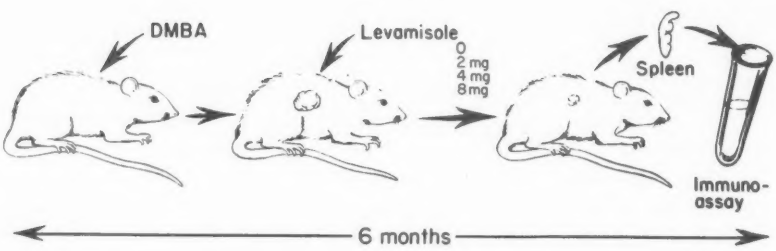

FIGURE 1.—Experimental design of the rat breast cancer studies.

For the clinical study, levamisole was administered in a randomized double-blind fashion to patients undergoing general surgical procedures at a dose of 150 mg/day on the day before surgery and for 2 days after surgery. The responses of their peripheral blood lymphocytes to vegetable mitogens prior to surgery and on Days 1, 3, 7, and 11 after surgery were measured. Thirteen patients were entered in the study, seven of whom received levamisole and six of whom received a placebo.

RESULTS

Figure 2 demonstrates the effect of different concentrations of levamisole on the responses of peripheral blood lymphocytes from normal donors to the three vegetable mitogens. Maximum augmentation of the response occurred at 0.6–0.8 μg/ml. At doses > 1.0 μg/ml, there was no augmentation and some suppression occurred.

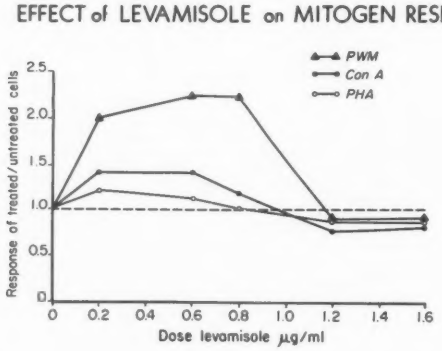

FIGURE 2.—Each point is the mean of 6 experiments.

The effect of different doses of levamisole on tumor growth in rats bearing breast cancer is illustrated in figure 3. Maximum inhibition of tumor occurred at a dose of 4 mg/kg, and at an 8-mg/kg dose no effect on the tumor was seen when compared with untreated controls. Figure 4 shows the mean diameter of the tumors at the end of the study compared with the dose of levamisole administered. Tumors were smallest in the group receiving the 4-mg/kg dose.

The responses of splenic lymphocytes of the rats at the end of the study are shown in figure 5. The maximal responses are seen in those rats receiving 2–4 mg/kg of levamisole, while those receiving 8 mg/kg did not differ from untreated controls.

The responses of peripheral blood lymphocytes from patients undergoing surgery and treated with levamisole during the perioperative period did not differ from those given a placebo, and thus the levamisole was ineffective in this experiment.

DISCUSSION

The studies described show that levamisole is able to augment human lymphocyte responses to mitogens in vitro and to inhibit breast cancer growth in the rat. Augmentation of in vivo lymphocyte responsiveness appears to correlate with the tumor inhibition in the rat. However, all these effects were shown to be dose-dependent, and at high doses the effects appear to be lost, with a potentially dangerous suppression of immune responses possibly occurring. If these studies are extrapolated to human clinical trials in the treatment of cancer, it would seem prudent to include some in vitro monitors of immune competence in the studies in order to be sure that augmentation of responses was being achieved in those patients receiving levamisole.

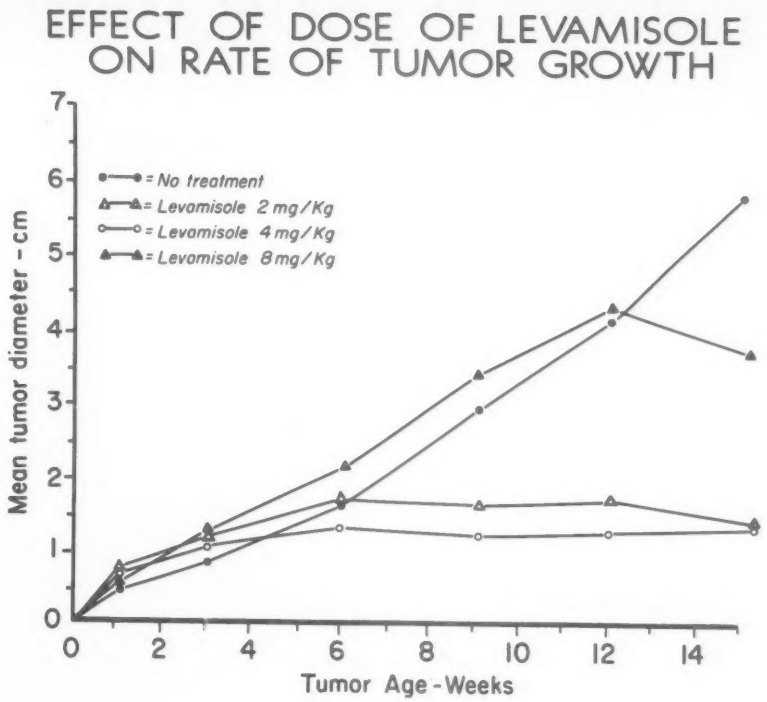

FIGURE 3.—Each point is the mean of the diameter of all tumors measured in the group of animals for each dose.

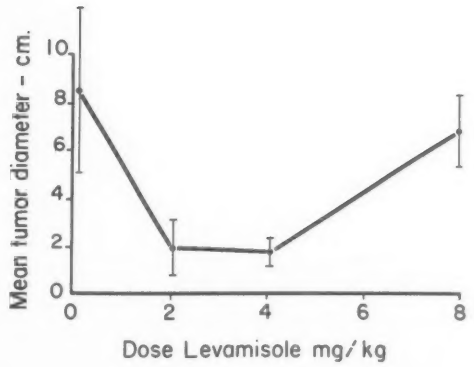

FIGURE 4.—Each point is the mean diameter of the tumors in each group 6 mos after the induction of breast cancer. Bars = standard deviation.

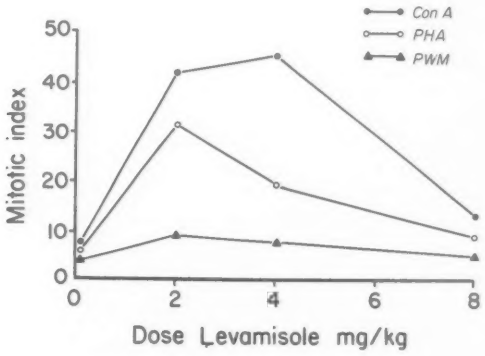

FIGURE 5.—Each point is the mean of 3–5 observations.

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Restorative Effects of Levamisole on Cell-Mediated Immune Responses Following Chemotherapy With Aniline Mustard in Mice Bearing ADJ-PC5 Plasmacytoma [1]

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SUMMARY

Restorative effects of levamisole on general and tumor-associated cell-mediated immune responses were investigated following aniline mustard (AM) chemotherapy of BALB/c mice bearing ADJ-PC5 plasmacytoma. Total eradication of tumor following AM chemotherapy resulted in severe depression of lymphoproliferative (LP) responses which recovered after a prolonged period of 4–6 weeks. During this time, spleen cells from these treated mice were shown to be generally depressed to T- and B-cell mitogens. Levamisole, an anthelmintic drug capable of enhancing depressed immune responses in mice and in man, was employed following AM chemotherapy in an attempt to restore immunocompetency. Administration of levamisole following AM had a significant effect on the ability of mice to resist rechallenge with ADJ-PC5 tumor and in tumor cell neutralization. The enhanced resistance to tumor cell challenge appeared to be associated with a faster recovery of the general T-cell immunocompetence as demonstrated in the LP assays among the mice receiving chemotherapy followed by adjuvant therapy. Levamisole, when administered alone to tumor-bearing mice, did not appear to possess a direct antitumor effect. In addition, levamisole did not potentiate cellular immunity to higher than normal levels in nonimmunodepressed mice. These results demonstrate the efficacy of levamisole as a restorative agent of the general and tumor-associated immunocompetency in the immunodepressed host.

[Cancer Treat Rep 62:1627–1635, 1978]

Neoplastic disease in animals and man is often accompanied by defective immune responses (1). Mineral oil-induced plasmacytomas represent a unique group of tumors in that they are induced in highly inbred BALB/c mice by the ip injection of mineral oil (2). We (3,4), as well as others (5–7), have shown that mineral oil-induced plasmacytomas of BALB/c mice possess distinct and/or common tumor-associated antigens (TAA).

Experimental evidence indicates that a variety of murine plasmacytomas induce a general depression in immunocompetency and that the degree of depression is correlated with the magnitude of tumor burden (4,8). Although it is unclear whether diminished immunocompetence precedes or is a result of progressively growing tumors, the high prevalence of neoplasia in congenital immune deficiency states and in immunosuppressed transplant recipients (9) suggests that depressed immunity contributes to the progression of malignant tumors. Thus, cancer cells might possibly be further eliminated by an immune-potentiator capable of enhanc-

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[4]We greatly appreciate the support and encouragement of Dr. Michael A. Chirigos, Division of Cancer Cause and Prevention, National Cancer Institute. We also thank Kathy Murphy, Carolyn Fox, and the Document Production and Control Unit of Litton Bionetics, Inc, for their assistance in the preparation of this manuscript.

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ing the activity of the reticuloendothelial system. However, one of the major side effects of treatments with anticancer agents is nonspecific immunosuppression. Restoration of a depressed immune system with immunopotentiating agents may increase the therapeutic efficacy when employed in conjunction with chemotherapy.

The anthelmintic drug, levamisole, used in conjunction with an effective remission-inducing drug (BCNU), produced long-term survivors in mice bearing leukemia in contrast to the groups of animals treated with either levamisole or BCNU alone (10). Further studies with experimental tumors and in man have suggested that levamisole acts to potentiate immune responses detected in vitro (11,12) and in vivo (10,13–15). More specifically, levamisole has been shown to stimulate the T-cell compartment of mouse spleen cells (16,17) and human peripheral blood lymphocytes (12). On the other hand, there are some conflicting reports which show that levamisole was not active in in vitro human lymphocyte transformation (18) and was not active in the treatment of several animal tumors (19,20). In addition, the effect of levamisole was dose-dependent; high doses suppressed rather than enhanced the immune response (21).

The experiments reported here were designed to study whether levamisole had any antitumor activity in BALB/c mice bearing transplantable plasmacytomas. More importantly, studies were conducted both in vivo by tumor transplantation rejection and tumor cell neutralization assays and by an in vitro lymphoproliferative (LP) assay to determine whether levamisole had immune restorative effects when used in combination with aniline mustard (AM).

MATERIAL AND METHODS

Mice

Male and female inbred BALB/c mice weighing 20–24 g (approximately 8–10 weeks old) were obtained from the production colonies of the National Institutes of Health, Bethesda, Md, and were used throughout these studies.

Tumor

The mineral oil-induced ADJ-PC5 plasmacytoma obtained from Dr. Michael Potter of the National Cancer Institute (NCI) has been passaged weekly by sc inoculation of 3×10^6 viable tumor cells suspended in Hanks' balanced salt solution into syngeneic BALB/c mice.

Drugs

AM was kindly supplied by the NCI. The agent was stored in a refrigerated dessicator and prepared fresh daily each time it was used. The drug was dissolved in heated commercially obtained corn oil (Mazola, Best Foods, Englewood Cliffs, NJ) and was administered ip in a single injection of 35 mg/kg of body weight.

Levamisole was a gift from Dr. David Klein of the NCI. The drug was dissolved in normal saline and administered ip in a single injection of 0.5 mg/kg of body weight. Cyclophosphamide, obtained from Mead Johnson Laboratories, Evansville, Ind, was dissolved in normal saline and injected ip at 180 mg/kg of body weight. BCNU was supplied by the NCI. This alkylating agent was dissolved in hydroxypropyl cellulose (0.3% solution) and administered sc in a single injection of 30 mg/kg of body weight.

Immunization

BALB/c mice were immunized with ADJ-PC5 by the method of Potter and Walters (22). Briefly, ADJ-PC5 tumor cells (2×10^5) were inoculated intradermally into mice; when the tumors reached 6–10 mm in size, these mice were treated ip with a single dose of 35 mg/kg of AM. Most of the tumors completely regressed in 10–14 days after AM chemotherapy. However, as we (4) have previously reported, both the general and tumor-specific cell-mediated immune (CMI) responses were severely depressed and this depression remained for 4–6 weeks before recovery of both T- and B-cell responses. Thus, levamisole was administered as an adjunct to AM chemotherapy with the rationale that levamisole would have a restorative effect on the cells of the reticuloendothelial system and, as such, recovery of the immunocompetency of these animals would occur at a faster rate when compared with AM chemotherapy alone.

Tumor Cell Challenge

One to four weeks after chemotherapy or chemotherapy followed by adjuvant therapy, mice were challenged by sc inoculation of 1×10^5 viable ADJ-PC5 tumor cells. All animals were palpated three times weekly to detect tumor development. Animals without tumors by Day 60 usually remained tumor-free.

Winn Tumor Cell Neutralization Assay

BALB/c mice that had been immunized intradermally with ADJ-PC5 tumor cells followed by AM treatment alone or in combination with levamisole

were employed as spleen cell donors in the cell neutralization assays. Spleen cell donors were used 1–4 weeks following AM-induced regression of tumors. Spleen cells were prepared in a single-cell suspension by gently teasing the cells from the spleen mass. The number of viable cells was determined by the trypan blue exclusion method. Viable spleen and tumor cells at ratios of 300:1 or 200:1 were incubated for 30 minutes at 37°C in a shaking water bath. The spleen-tumor cell mixtures containing the approximate number of cells to induce tumors in approximately 90% of the inoculated hosts were injected sc into BALB/c recipients.

Microculture Lymphoproliferative Assay

A microculture (200 μl) LP assay that utilized general mitogens was employed to stimulate the proliferation of immunocompetent spleen cells. This assay was previously described by Dean et al (23). Viable spleen cells (2×10^5) from tumor-bearing and drug-treated or normal mice were cultured in four replicate flat-bottom wells of a microtiter plate (Falcon Plastics, Oxnard, Calif) fitted with a Linbro lid (No. 55, Linbro Plastics, Vineland, NJ). The culture medium consisted of RPMI-1640 (Gibco, Grand Island, NY) supplemented with 25 mM Hepes buffer, 5% heat-inactivated (56°C for 30 minutes) fresh-frozen human AB serum (Irvine Scientifics, Calif), 1% glutamine, and 50 μg of gentamicin/ml. Purified phytohemagglutinin (PHA) (0.25 μg/well; Burroughs Wellcome Co, Research Triangle Park, NC), concanavalin A (Con A) (0.25 μg/well; Calbiochem, San Diego, Calif), T-cell mitogens, and *Escherichia coli* lipopolysaccharide (LPS) (5 μg/well; Difco Laboratories, Detroit, Mich), a B-cell mitogen, were added to each culture. Six hours before culture termination, 1.0 microcurie of ^{3}H-TdR (New England Nuclear Corp, Boston, Mass; specific activity, 6.7 curies/mm) was added to each culture. After 72 hours of culture, the cells were harvested with a multiple sample precipitator (Biomedical Research and Development Laboratories, Inc, Rockville, Md) and the amount of ^{3}H-TdR incorporation into 5% cold trichloroacetic acid-insoluble material was determined by liquid scintillation spectroscopy. These data were expressed as the mean cpm of the four replicates that usually had standard errors of $\leq 5\%$. Mitogen data from spleen cell cultures of tumor-bearing and drug-treated mice were expressed as percent suppression. The formula used for percent suppression was the cpm of the mitogen-treated cultures minus medium control cultures (net cpm) of the tumor-bearing and/or drug-treated mice divided by the net cpm of drug-treated or normal mice minus 1×100.

RESULTS

To determine whether progressively growing ADJ-PC5 plasmacytomas were capable of suppressing the general CMI responses of tumor-bearing mice, experiments were conducted to study spleen cells from ADJ-PC5-bearing mice to be stimulated by the mitogens PHA, Con A, or LPS in microculture LP assays. The proliferative responses to PHA, Con A, and LPS of spleen cells from BALB/c mice bearing medium to large (15–30 mm in diameter) ADJ-PC5 tumors were strongly suppressed compared to the non-tumor-bearing control mice studied in parallel tests (fig 1). The LP response of spleen cells was markedly reduced to PHA (50%), Con A (67%), and LPS (20%). This suppression to PHA, Con A, and LPS remained during progressive tumor growth. In mice with large tumors, the PHA, Con A, and LPS responses were further reduced to 42%, 70%, and 53% respectively. The results demonstrate that the proliferation to both T- and B-cell mitogens of spleen cells in ADJ-PC5 tumor-bearing mice was severely reduced.

We next assessed the general LP responses of ADJ-PC5-bearing mice after removal of the tumor load by AM chemotherapy. The lymphocyte function of ADJ-PC5 tumor-bearing mice whose tumors had completely regressed after AM therapy was studied by means of LP assays with PHA and LPS. After AM treatment, spleen cells from tumor-regressed mice were strongly suppressed in their proliferative response to PHA for at least 14 days (table 1). This suppression of the general T-lymphocyte function in the response to PHA after tumor regression and/or AM therapy was slowly restored by Day 35.

Parallel LP experiments demonstrated that the response of spleen cells to the B-cell mitogen LPS in tumor-bearing mice similarly recovered after regression of tumor by drug therapy (table 2). On Day 7 after drug therapy, the response to LPS was still quite reduced (87%), but by Day 14 a partial recovery of the LPS response was observed (33% reduction). Spleen cells from tumor-bearing mice on Day 28 after AM therapy had a 7% greater stimulation to LPS than did spleen cells from normal mice treated with AM.

To ascertain whether levamisole was active in the treatment of ADJ-PC5 plasmacytoma, BALB/c mice were inoculated sc 1×10^6 viable ADJ-PC5 cells. When the tumors reached 6–8 mm in diameter, mice were treated with levamisole, AM, BCNU, or cyclophosphamide. The results are presented in table 3. A single treatment with AM (35 mg/kg), BCNU (30 mg/kg), or cyclophosphamide (180 mg/kg) increased the median survival time (MST) of the plasmacytoma-bearing mice > 200% over the un-

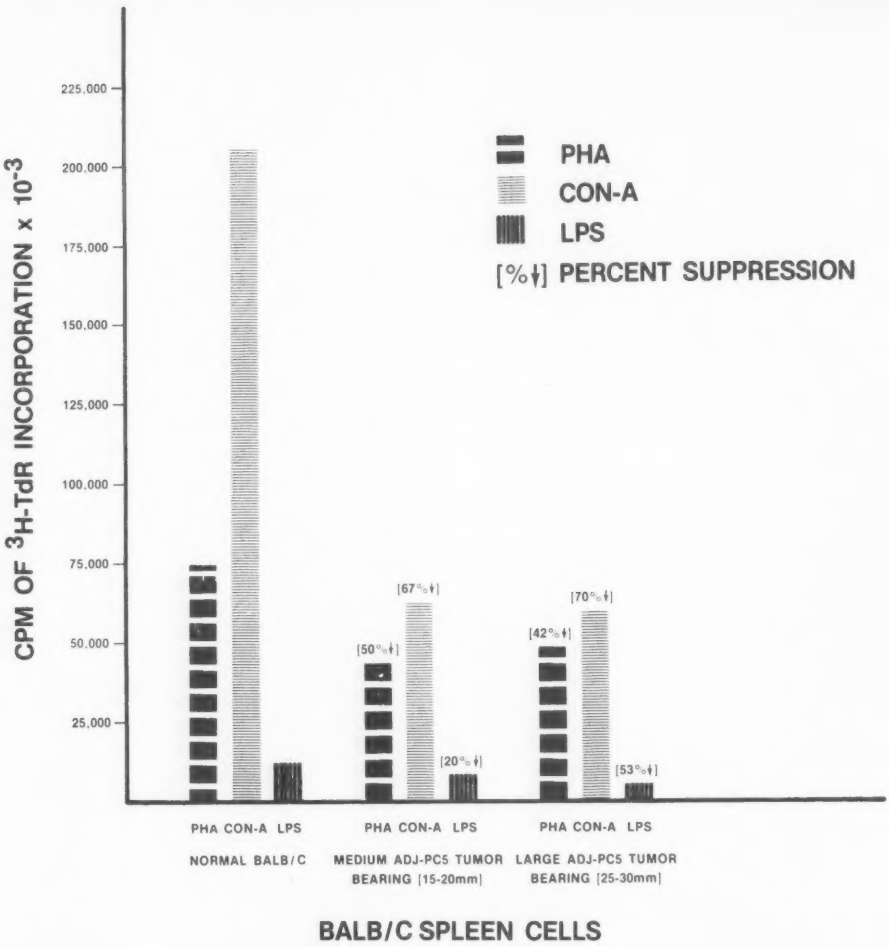

FIGURE 1.—LP responses to the general mitogens of spleen cells from medium and large ADJ-PC5 tumor-bearing mice. The data are expressed as net cpm and % suppression in parentheses (the cpm of the mitogen-treated cultures minus medium control values [net cpm] of the tumor-bearing or treated mice divided by the net cpm of normal mice minus 1 × 100). Optimum blastogenic concentrations of PHA (0.25 μg/well), Con A (0.25 μg/well), and LPS (5.0 μg/well) were used in spleen cells pooled from 3 mice in each group and assayed on the same day.

treated controls. The MST of the plasmacytoma-bearing mice that were treated with levamisole (0.5 mg/kg) was similar to that of the untreated controls. Thus, it appeared that treatment with levamisole had no antitumor activity against ADJ-PC5 plasmacytoma.

It has been reported that levamisole augmented BCNU chemotherapy against a murine lymphoid leukemia (10), and that the mechanism of action of the drug appeared to be through stimulation of T-cell proliferation or maturation (16,17). We were interested in determining whether levamisole might restore the CMI response following AM chemotherapy in mice bearing ADJ-PC5 plasmacytoma. Initially, we evaluated the possible stimulatory effect of levamisole in LP assays to the general mitogens with spleen cells from normal BALB/c mice (table 4). Essentially, similar LP results were observed with spleen cells from normal BALB/c mice either untreated or treated with levamisole at 3 or 0.3 μg/ml. When cells were treated with levamisole at 30 μg/ml, the LP responses to both the T- and B-cell mitogens were quite suppressed.

We next examined pooled spleen cells from mice

TABLE 1.—Recovery of the PHA response of spleen cells from ADJ-PC5 tumor-bearing BALB/c mice after complete remission with AM

Days after AM therapy	Mean tumor diameter (mm)	Net cpm of ^{3}H-TdR incorporation			
		Normal untreated mice	Normal mice treated with AM*	Mice bearing ADJ-PC5 and treated with AM*	% suppression in tumor-bearing and AM-treated mice
0	10	162,351†	—	17,772‡	89% ↓
7	5	110,980	26,995‡	22,945‡	15% ↓
14	0§	99,263	47,534‡	28,004‡	41% ↓
21	0§	114,652	105,650	92,852	12% ↓
35	0§	168,395	166,580	152,572	8% ↓

* Mice were treated with AM (35 mg/kg) on Day 0. The Day-0 LP responses were determined in untreated mice from each group.
† Spleens were pooled from 3 normal untreated, 3 normal treated, or 3 tumor-bearing and treated BALB/c mice/group; values = the mean of 4 cultures.
‡ Significantly different from response in normal untreated mice at $P < 0.05$.
§ Complete regression of ADJ-PC5 tumor.

TABLE 2.—Recovery of the LPS response of spleen cells from ADJ-PC5 tumor-bearing BALB/c mice after AM chemotherapy

Days after AM therapy	Mean tumor diameter (mm)	Net cpm of ^{3}H-TdR incorporation			
		Normal untreated mice*	Normal mice treated with AM†	Mice bearing ADJ-PC5 and treated with AM†	% suppression in tumor-bearing and AM-treated mice
0	10	14,806	—	6764‡	54% ↓
7	5	13,608	4560‡	600‡	87% ↓
14	0§	9736	5448‡	3656‡	33% ↓
28	0§	10,077	11,157	11,988	7% ↑

* Spleens from 3 normal untreated, 3 normal treated, or 3 tumor-bearing and treated BALB/c mice/group were used.
† Mice were treated with AM (35 mg/kg) on Day 0. The Day-0 LP responses were determined in untreated mice from each group.
‡ Significantly different from controls at $P < 0.05$.
§ Complete regression of ADJ-PC5 tumor.

bearing ADJ-PC5 treated with AM alone or in combination with levamisole in LP assays with mitogens. These experiments were performed to determine whether levamisole was capable of restoring the general CMI responses of the tumor-bearing, AM-treated mice (table 5). Whereas spleen cells from ADJ-PC5 tumor-bearing mice that were treated with AM alone were markedly suppressed to PHA (49%) and LPS (87%), the LP responses to PHA of spleen cells from ADJ-PC5 tumor-bearing mice treated with AM in combination with levamisole were restored by Day 14 after AM therapy. In contrast, the LP response to LPS of these tumor-bearing and AM plus levamisole-treated mice was essentially unaltered by levamisole treatment. Thus, the restorative effect of levamisole on these tumor-bearing and AM-treated mice appeared to be in the T-cell compartment. The recovery of the PHA response by Day 14 after AM and levamisole therapy was 94% of the control responses and by Day 21 there was a 4% greater stimulation to the mitogens compared to spleen cells from normal untreated mice.

To determine if the restorative effect of the general spleen cell functions as assayed by LP was also accompanied by a general recovery of the cell population's ability to react against TAA of ADJ-PC5, tumor transplantation rejection and tumor cell neutralization assays were performed with ADJ-PC5 cells. In the tumor-rejection studies, mice were randomly selected from tumor-bearing and drug-treated and from normal (nontumor and non-treated) control groups and secondarily challenged on Days 7, 14, 21, and 28 after AM-levamisole therapy. The results are presented in table 6. ADJ-PC5-bearing mice treated with AM in combination with levamisole strongly resisted tumor cell challenge by Day 14 after AM therapy, whereas AM-alone treated or untreated control mice developed tumors. This combined effect of AM chemotherapy plus levamisole remained through Day 21 after AM therapy at which time only two of 12 mice had developed tumors. Further, by Day 28 solid protection was observed in the tumor-bearing, AM-treated mice as well as in the tumor-bearing mice treated with the combination of AM and levamisole.

TABLE 3.—Effect of treatment with levamisole in mice bearing progressively growing ADJ-PC5 plasmacytoma*

Treatment	Dose (mg/kg)	MST† (days)	Death range (days)	% ILS‡
None	—	28.5	13–35	—
Levamisole	0.5	27.5	14–36	—
AM	35.0	> 60.0	—	> 200 (10)§
BCNU	30.0	> 60.0	—	> 200 (10)
Cyclophosphamide	180.0	> 60.0	14,34	> 200 (8)

*BALB/c mice were inoculated sc with 1×10^6 viable ADJ-PC5 tumor cells followed by a single treatment of levamisole, AM, BCNU, and cyclophosphamide when the tumors were 6–10 mm in diameter (approximately Day 10).

†MST = median survival time.

‡ILS = increase in MST over controls.

§Nos. in parentheses are 60-day survivors of 10 BALB/c mice/group.

TABLE 4.—Effect of levamisole on LP responses of normal BALB/c spleen cells

In vitro treatment of spleen cells	Net cpm of ^{3}H-TdR incorporation		
	PHA (0.25 μg/well)	Con A (0.5 μg/well)	LPS (5 μg/well)
None	27,565*	54,003	7554
Levamisole, 0.3 μg/ml	30,587	55,018	7898
Levamisole, 3.0 μg/ml	30,522	49,732	9744
Levamisole, 30 μg/ml	7492†	10,231†	2414†

*Values = mean cpm experimental minus mean cpm control (net cpm) of 4 cultures of spleens pooled from 3 normal BALB/c mice.

†Net cpm significantly lower than LP response in nontreated spleen cell cultures at $P < 0.05$.

TABLE 5.—Restorative effect of levamisole treatment on LP responses of spleen cells from ADJ-PC5 tumor-bearing mice after AM therapy and tumor regression

Days after AM therapy	Normal untreated BALB/c mice*	ADJ-PC5 tumor-bearing AM-treated mice†	% suppression	ADJ-PC5 tumor-bearing AM-treated mice‡ (levamisole therapy on Day 10)	% suppression	Significant restorative effect§
	PHA					
7	165,832	85,335	49% ↓	Not applicable	Not applicable	Not applicable
14	62,704	42,962	31% ↓	58,997	6% ↓	$P < 0.05$
21	209,031	126,288	38% ↓	218,062	4% ↑	$P < 0.05$
	LPS					
7	8333	1042	87% ↓	Not applicable	Not applicable	Not applicable
14	15,791	9685	39% ↓	9526	39% ↓	Not significant
21	12,333	8670	30% ↓	9603	22% ↓	Not significant

*Spleens from 3 normal untreated BALB/c mice were used.

†BALB/c mice were inoculated intradermally with 1×10 viable ADJ-PC5 tumor cells followed by ip treatment with AM (35 mg/kg) when the tumors were 6–10 mm in diameter.

‡Levamisole (0.5 mg/kg) given ip to ADJ-PC5-bearing mice on Day 10 after AM treatment.

§Significant difference of net cpm of LP response in AM plus levamisole-treated and AM-treated mice alone determined by Student's t test.

TABLE 6.—Tumor rejection of ADJ-PC5 plasmacytoma by mice bearing ADJ-PC5 tumor and treated with AM alone or in combination with levamisole

Mice challenged*	Days after AM chemotherapy			
	7	14	21	28
Normal untreated mice	12/12 (15)†	10/12 (16)	11/12 (16)	12/12 (14)
Mice bearing ADJ-PC5 tumor and treated with AM‡	10/12 (17)	8/10 (18)	8/12 (14)	4/12 (18)
Mice bearing ADJ-PC5 tumor and treated with AM‡ and levamisole (Day 10)§	—	1/10	2/12 (20)	0/12

*BALB/c mice were secondarily challenged with 1×10^5 viable ADJ-PC5 cells on Days 7, 14, 21, and 28 after AM treatment.

†No. of mice developing palpable tumors/No. of mice inoculated (mean latent period in days). Last day of observation was Day 60.

‡BALB/c mice were inoculated intradermally with 1×10^6 viable ADJ-PC5 tumor cells followed by ip treatment with AM (35 mg/kg) when the tumors were 6–10 mm in diameter.

§Levamisole (0.5 mg/kg) given ip on Day 10 after AM treatment.

In the tumor cell neutralization assays, spleens from untreated or tumor-bearing and drug-treated mice were incubated with ADJ-PC5 cells (table 7). Whereas spleen cells from normal mice or mice bearing ADJ-PC5 and treated with AM failed to neutralize ADJ-PC5 tumor cells in assays conducted on Days 7 and 14 after AM therapy, spleen cells from ADJ-PC5 tumor-bearing mice treated with levamisole on Day 10 demonstrated strong neutralization of ADJ-PC5 tumor cells. However, by Day 21, tumor cell neutralization was obtained in mice bearing ADJ-PC5 treated with AM. Strong neutralization was also observed on Day 21 with spleen cells from ADJ-PC5 tumor-bearing animals that received the combination treatment of AM and levamisole. Again, these identical pools of spleen cells were shown to have strongly restored responses to PHA at 14 and 21 days after AM and levamisole treatments.

DISCUSSION

Studies from our laboratories (4) as well as others (8) have demonstrated that mice bearing plasmacytomas have a decreased capacity to induce a primary antibody response. We (4) have also shown that the CMI responses as measured by LP assay became strongly depressed as the plasmacytoma tumor burden increased.

Recovery of the general CMI response following complete tumor regression with AM chemotherapy demonstrated a progressive return of the T-cell (PHA) response. However, restoration of LP reactivity was a very slow process requiring 4–6 weeks. In addition, the B-cell responses were restored by 4 weeks following treatment with AM.

The mechanism of this general immune depression of spleen cells by progressive growth of the ADJ-PC5 tumor appears to be correlated with the induction of adherent suppressor cells (Padarathsingh et al, manuscript in press). Moreover, these animals must be rechallenged with the viable tumor before they demonstrate protective antitumor immunity in tumor cell neutralization assays. Thus, because of the uniqueness of this model, we were able to study the immunorestorative role of levamisole in both general CMI competency and antitumor immunity.

Levamisole, a widely used anthelmintic drug, appears to improve host defense mechanisms by stimulating phagocytes and lymphocytes when their functions are depressed (14,24). However, in contrast to other immunostimulants, levamisole does not stimulate immunity above the normal level (25) and the direct effect of the drug on experimental mouse tumors is highly controversial (15,19,20). However, when the drug was employed as an adjunct to cytoreductive therapy, the number of long-term survivors bearing a transplantable leukemia was markedly increased (10).

The results of our studies with normal spleen cells indicated that levamisole in concentrations ranging from 0.3 to 3.0 μg/ml utilized in vitro did not modulate the LP response to PHA or LPS, while the highest concentration (30 μg/ml) was found to suppress LP reactivity. These results were similar to those in studies reported by Hokama et al (11) who likewise observed depressed LP responses at higher concentrations of levamisole but not at lower concentrations. In contrast, Lichtenfeld et al (12) observed enhanced LP reactivity at 33 μg/ml of levamisole. Further, Sampson and Lui (21) observed suppressed LP responses in the mixed leukocyte culture at 3 μg/ml of levamisole. The interpretation

TABLE 7.—Winn tumor cell neutralization of ADJ-PC5 plasmacytoma by spleen cells from mice bearing ADJ-PC5 tumor and treated with AM alone or in combination with levamisole

Source of spleen cells	Ratio of spleen cells:tumor cells	Days after AM chemotherapy		
		7	14	21
Normal untreated mice	300:1	10/10 (15)*	11/12 (16)	11/12 (16)
	200:1	10/10 (16)	12/12 (15)	12/12 (14)
Mice bearing ADJ-PC5 tumor and treated with AM†	300:1	10/10 (15)	10/12 (15)	8/12 (16)
	200:1	10/10 (16)	12/12 (15)	10/12 (15)
Mice bearing ADJ-PC5 tumor and treated with AM† and levamisole (Day 10)‡	300:1	—	4/12 (18)	4/12 (20)
	200:1	—	6/12 (17)	6/12 (16)

*No. of mice developing palpable tumors/No. of mice inoculated (mean latency period of tumor detection). Last day of observation was Day 60.

†BALB/c mice were inoculated with 1×10^6 viable ADJ-PC5 tumor cells followed by ip treatment with AM (35 mg/kg) when tumors were 6–10 mm in diameter.

‡Levamisole (0.5 mg/kg) was given ip to ADJ-PC5-bearing mice on Day 10 after AM treatment.

of these data from different laboratories is compounded by apparent differences in either sensitivity of lymphocytes from different species to the restorative effects of levamisole or differences in the LP assay between laboratories.

The effectiveness of a single inoculation of levamisole after AM therapy was demonstrated in the restoration of the mitogenic responses to the general T-cell mitogen (PHA) and in the rejection of syngeneic ADJ-PC5 plasmacytoma cells in tumor rejection and tumor cell neutralization studies. An injection of 0.5 mg/kg of levamisole to ADJ-PC5 tumor-bearing mice on Day 10 after AM therapy resulted in long-term survivors and cured animals as compared to untreated controls or ADJ-PC5 tumor-bearing mice receiving AM alone and rechallenged with ADJ-PC5 cells on Days 14 and 21 after AM treatment. In tumor cell challenge experiments conducted on Day 28, we observed good protection in the group treated with AM alone as well as in the group receiving the combination treatment of AM plus levamisole.

These studies confirm the findings of Woods et al (17) and Renoux et al (16) and extend the data concerning the ability of levamisole to restore T-cell responses in immunologically depressed hosts. Previous studies from our laboratory (3) have shown that T-cells are required for rejection of plasmacytoma cells in the syngeneic host. Thus, levamisole apparently acts by possibly restoring more rapidly the effector arm of the CMI response. The increase in survival of animals receiving AM plus levamisole could be due to immune stimulation as a direct result of the fast and progressive recovery of the general CMI responses of the ADJ-PC5 tumor-bearing and drug-treated depressed host. These results of antitumor studies correlate well with the recovery of general CMI competence as measured in vitro using the LP assay. The results of these studies therefore indicate that levamisole appears to facilitate restoration of the immune status in immunodepressed mice.

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☆U.S. GOVERNMENT PRINTING OFFICE: 1979 281-225/8 1-3

Effect of Levamisole on Metabolism of Phagocytic Cells

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SUMMARY

The effect of levamisole (LMS) on glucose metabolism was studied using a protozoan phagocytic model and human leukocytes. At concentrations of > 10 μg/ml, LMS inhibited glucose metabolism in the protozoan phagocytic model. Glucose metabolism in both the phagocytic model and normal peripheral blood leukocytes was, however, minimally altered when exposed to levels of LMS of < 10 μg/ml. In contrast, myeloblasts from a child with leukemia seemed to have increased metabolic activity and 5 μg/ml of LMS markedly reduced the glucose metabolism. These preliminary studies suggest that LMS can alter glucose metabolism of certain cells and that some malignant cells may be directly inhibited metabolically by LMS.

[Cancer Treat Rep 62:1637–1640, 1978]

In previously reported studies, we demonstrated that immunopotentiation therapy was effective against both bacterial and viral pathogens in suckling rats (1,2). In vivo studies suggested that protection against bacterial invasion was at least in part mediated through enhanced polymorphonuclear leukocyte activity (1). Further studies showed that levamisole (LMS) increased peroxidase activity in suckling rat spleen cells, thus suggesting that LMS might alter the intracellular metabolism of phagocytes (3).

In our initial studies of the effect of LMS on cellular glucose metabolism, we decided to evaluate the ciliated protozoan, *Tetrahymena pyriformis*, to determine if this might be a suitable model for phagocyte research, since these organisms have a glycolytic pathway and hexose monophosphate shunt (HMPS) similar to that found in human leukocytes and they can be easily controlled and manipulated in the laboratory.

Isolated mitochondrial preparations showed that LMS both inhibited and stimulated enzymes of the citric acid cycle (3). When analyzing these data, we were not sure what effect LMS might have on the metabolism of the intact phagocytic model or human phagocytic cells. To answer these questions, temperature-synchronized cultures of *T. pyriformis* or peripheral blood leukocytes were grown in the presence of various concentrations of LMS. Myeloblasts from a child with acute myelogenous leukemia were also studied to determine the effect of LMS on abnormal or immature phagocytes.

MATERIALS AND METHODS

T. pyriformis Metabolism

The organisms were grown in a 2% proteose-peptone-liver extract medium (4). The cells were incubated with shaking at 29°C until the population reached 150,000–200,000/ml. For experiments with synchronized cells, the method of Scherbaum and Zeuthen (5) was utilized. Temperature synchronization was initiated when the population reached 80,000–100,000/ml.

The protozoa (1-ml aliquots) were transferred to plastic scintillation vials where they were placed uncapped in a 5% CO_2 atmosphere at 37°C for 20 minutes. Hanks' balanced salt solution (0.9 ml) containing the appropriate concentration of LMS and/or heat-killed staphylococci was added, and the cells were incubated an additional 15 minutes.

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Human Leukocyte Metabolism

Heparinized venous blood was collected and allowed to settle at room temperature for 2–3 hours. The leukocyte-rich plasma was removed and diluted with Hanks' solution to give a final leukocyte concentration of approximately 10^7 cells/ml. Differential cell counts consistently showed > 90% of the peripheral venous cells to be neutrophils, or in the case of the leukemic patient, > 90% of the cells were myeloblasts.

One-milliliter aliquots were distributed to plastic scintillation vials and were placed uncapped in a 5% CO_2 atmosphere at 37°C for 20 minutes. An additional 0.9 ml of Hanks' solution containing the appropriate concentration of LMS and/or heat-killed staphylococci was added and was incubated for an additional 15 minutes.

The study period was then initiated by adding 0.1 microcurie of labeled glucose-1-^{14}C or glucose-6-^{14}C (0.1 microcurie/0.1 ml in H_2O) and sealing the vials with scintillation caps containing two layers of Whatman No. 1 filter paper wetted with 0.1 ml of hyamine hydroxide. The sealed vials were incubated at 37°C for 60 minutes and the reaction was stopped by adding 1.0 ml of 5% trichloroacetic acid. The vials were immediately recapped and allowed to sit at 37°C for an additional 30 minutes, after which time the filter paper was removed and counted.

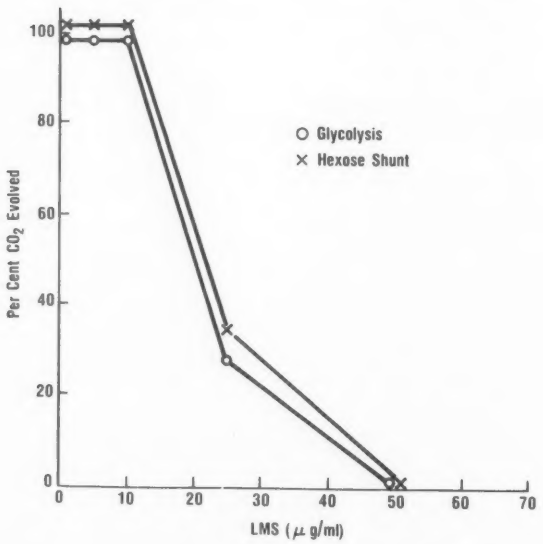

FIGURE 1.—Effect of LMS on CO_2 production from glucose-1-^{14}C (HMPS) or glucose-6-^{14}C (glycolysis) in *T. pyriformis*.

RESULTS

T. pyriformis

Cellular glucose metabolism was measured by adding glucose labeled with carbon 14 at either the 1 or 6 position to *Tetrahymena* cultures and measuring the production of labeled CO_2 during 60 minutes of incubation. LMS did not alter glucose metabolism significantly until concentrations > 10 μg/ml were used; no CO_2 evolution was recorded during the study period at 50-μg/ml LMS concentrations (fig 1).

To determine if stimulation of the HMPS with bacteria might alter the effect of LMS, heat-killed staphylococci were added to the cultures (fig 2). Little inhibition was seen at 10 μg/ml of LMS, but at 25 and 50 μg/ml, there was a marked reduction of glucose metabolism. Since the addition of heat-killed staphylococci did not increase glucose metabolism through the HMPS, as in human phagocytes, this protozoan was not an ideal model to study phagocyte metabolism.

Human Leukocytes

Therefore, we began to study the effect of LMS on the neutrophil HMPS. The addition of heat-killed

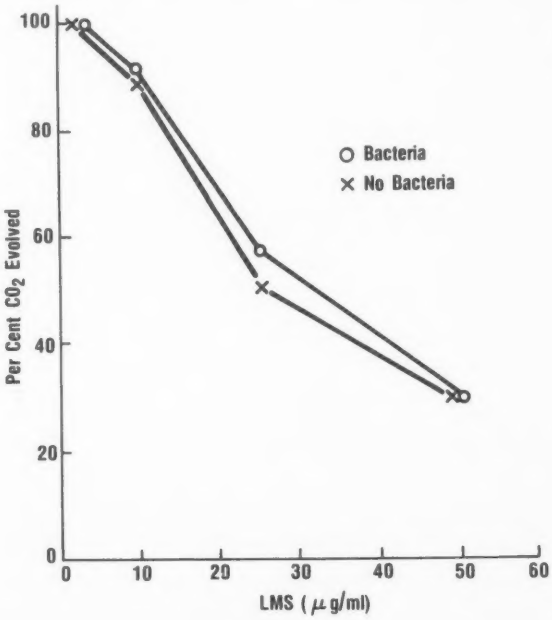

FIGURE 2.—Effect of LMS on CO_2 production from glucose-1-^{14}C in *T. pyriformis* incubated in the presence of heat-killed *Staphylococcus aureus*.

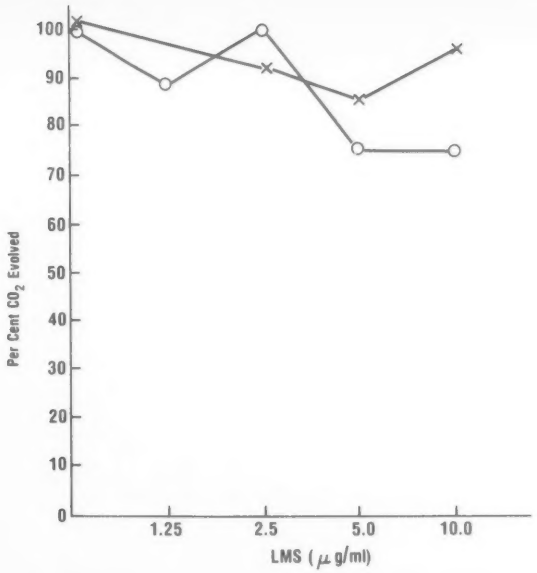

FIGURE 3.—Effect of LMS at various concentrations on CO_2 production from glucose-1-^{14}C in peripheral blood leukocytes (2 separate studies).

staphylococci to leukocyte cultures consistently produced a marked increase in metabolic activity via the HMPS as measured by CO_2 production from glucose labeled at the 1 carbon position (table 1). LMS did not significantly alter the metabolism of glucose through the HMPS. These studies were repeated at various concentrations of LMS where we previously found activity in other in vitro studies but we again found little effect on C-1 labeled glucose metabolism. Although some slight reduction of activity was seen in the presence of LMS, the normal CO_2 production was consistently $> 70\%$ (fig 3). This is not too surprising, however, since LMS usually had a minimal effect on normal cells.

We, therefore, decided that immature leukocytes such as myeloblasts might provide a good cell type to study. Our hypothesis was that since these are immature cells they might also demonstrate altered metabolic activity when exposed to LMS similar to

TABLE 1.—Effect of heat-killed staphylococci and LMS on metabolism of glucose-1-^{14}C to CO_2 in normal adult leukocytes

	cpm evolved in 60 mins	
Heat-killed staphylococci	No LMS	5 μg/ml of LMS
0	202	216
+	765	670

TABLE 2.—Effect of LMS on metabolism of glucose-1-^{14}C and glucose-6-^{14}C to CO_2 in a leukemic adolescent

	cpm evolved in 60 mins	
Glucose substrate	No LMS	5 μg/ml of LMS
Glucose-1-^{14}C	563	69
Glucose-6-^{14}C	520	161

the suckling rat spleen cell studies. We had the opportunity to study myeloblasts from a young boy with acute myelogenous leukemia. He had a wbc count of 30,000/mm^3 with 95% blast cells. The patient had received no chemotherapy prior to obtaining the cells.

The peripheral blood cells from the leukemic patient had a nonstimulated CO_2 production similar to those values seen with bacterial stimulation of normal leukocytes and higher than those we normally had seen with unstimulated peripheral blood cells (table 2). We did not have enough cells to include studies with the addition of staphylococci; however, we did look at the regular glycolytic pathway (CO_2 produced from C-6 labeled glucose) and it was extremely active also, as if the cells were hypermetabolic. Much to our surprise, the addition of 5 μg/ml of LMS dramatically decreased CO_2 production in these cells and, in fact, brought the level of C-1 labeled glucose metabolism to approximately the level of normal adult leukocytes.

DISCUSSION

Although LMS has been shown to enhance cell-mediated immunity and to protect suckling rats from both viral and bacterial pathogens, the mechanism of action of LMS remains unknown. Previous studies using suckling rat spleen cells have shown that LMS increases peroxidase activity in 2-day-old rat cells, while spleen cells from older animals that were not susceptible to bacterial challenge had normally higher levels of peroxidase and were not affected by the addition of LMS (3). At concentrations of < 10 μg/ml, LMS had little effect in vitro on glucose activity through the HMPS in either normal leukocytes or *T. pyriformis*. Since *Tetrahymena* did not increase HMPS activity with the ingestion of bacteria in a fashion similar to normal human phagocytes, this protozoan was not considered a good model for further studies of HMPS activity.

The fact that glucose metabolism of normal human leukocytes was not significantly altered by LMS (at concentrations of < 10 μg/ml) is not too surprising, since in our previous studies only imma-

ture cells demonstrated enhanced activity in the presence of LMS. We were interested, therefore, to study immature or undifferentiated cells from patients with acute myeloblastic leukemia to determine if LMS might alter the glucose metabolism in these cells. Of interest was the finding that myeloblastic cells seemed to be metabolically hyperactive. The evolution of labeled CO_2 was 2–3 times that recorded for nonstimulated peripheral blood leukocytes. Furthermore, the addition of 5 μg/ml of LMS (a level similar to that achieved in patients) markedly reduced glucose metabolism.

Cells were not available to repeat these studies since the patient immediately received chemotherapy after we received his cells for study, but we did study his cells that were placed in tissue culture. When his cells were incubated with LMS at 0.1, 1.0, and 10.0 μg/ml and compared to untreated control cultures, there was a small, but consistent, stepwise decrease in tritiated thymidine uptake after 24 hours, again suggesting decreased activity in the presence of increasing amounts of LMS.

Although these studies are very preliminary, they raise several critical questions about the effects of LMS on phagocytes and malignant cells. First, are some malignant cells hypermetabolic and, if so, could LMS regulate or inhibit glucose metabolism in these cells? Can LMS directly inhibit the growth of some malignant cells, particularly monoblasts or myeloblasts? If LMS could regulate the metabolism of some malignant cells, could it be used to synchronize the growth of these cells and make them more susceptible to antitumor chemotherapy in a combined therapy protocol? To effectively use LMS in the treatment of neoplasia, it seems essential to analyze the effect of LMS not only on the host's immunologic system, but also on the malignant cells themselves.

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Effects of Bacillus Calmette-Guérin and Levamisole on Immune Responses in Young Adult and Age-Immunodepressed Mice [1]

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SUMMARY

The immunomodulating effects of bacillus Calmette-Guérin (BCG) and levamisole were tested in young adult mice after a single administration and in 12-month-old mice after continuous administration. In young mice, BCG was shown to activate macrophages, to potentiate antibody responses and delayed hypersensitivity reaction, to increase antibody-dependent cell-mediated cytotoxicity, and to induce nonspecific suppressor cells. Levamisole was not able to modify any of these immune responses in young mice. The action of these adjuvants differed markedly when tested in age-immunodepressed mice. BCG was found to be strongly immunosuppressive on antibody formation and induced suppressor cell activity in the spleen. Moreover, the survival of immunodepressed mice continuously treated with BCG was shortened in comparison to untreated aged mice. In contrast, levamisole acted as an immunorestoring agent because it strongly stimulated the antibody response compared to aged controls and did not induce suppressor cell population. The survival of levamisole-treated mice was prolonged when compared to untreated aged mice. When the surviving mice were killed and autopsied at the age of 24 months, the incidence of spontaneous tumors was significantly lower in the group of mice treated by levamisole.

[Cancer Treat Rep 62:1641–1650, 1978]

We investigated the effects of bacillus Calmette-Guérin (BCG) and levamisole with respect to their immunostimulating and immunorestoring capacities in both young adult and age-immunodepressed mice. In the first instance, we were interested in analyzing the mode of action of these agents after single different doses, whereas in the aged animals, we looked for the possible immunorestoring properties after repeated administration of these substances.

BCG is presently one of the most studied immunostimulating agents, but the precise mode of its action is still not known. We have shown that BCG was able to potentiate humoral responses and delayed-type hypersensitivity reaction and to activate peritoneal macrophages (1). The dose capable of producing these stimulatory effects was also able to evoke an antitumor activity in vivo (2) while, concomitantly, suppressor cells were detected in the spleen of BCG-treated animals (1,3).

Levamisole has also been the object of many studies. It has been shown to be clinically effective in some disorders, such as sarcoidosis, rheumatoid arthritis, and certain types of infections (4,5). Clinical trials in neoplastic diseases provided opposing results (6,7). Laboratory experiments have also produced divergent data (8–10). It is generally believed that levamisole acts as an immunorestorator rather than as an immunostimulating agent.

In this study, we report the effects of these substances on macrophage activation, antibody production, delayed hypersensitivity reaction, suppressor cell induction, and antibody-dependent cell-mediated cytotoxicity, in both normal and in immunodepressed animals.

[1] Supported by grant 51.77.83 from INSERM.

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MATERIALS AND METHODS

Mice.—Two- and 12-month-old (DBA/2 × C57BL/6)F_1 female mice, obtained from IFFA CREDO Laboratories (Lyon, France), were used.

BCG.—A fresh preparation of BCG, < 7 days old and containing 7×10^6 viable organisms/mg, was supplied by the Pasteur Institute (Paris, France).

Levamisole.—The levamisole used was the L-isomer of 2,3,5,6-tetrahydro-6-phenyl-imidazo-(2,1-*b*)-thiazole obtained from Janssen Pharmaceutica Co (Brussels, Belgium).

Macrophage cytostatic test.—Peritoneal cells obtained by washing the peritoneal cavity and exudates from four mice in each group were pooled. Five × 10^4 EAkR lymphoma cells (C57BL/6) in 0.2 ml of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) were incubated for 24 hours at 37°C on macrophage monolayers in culture microplates (the macrophage to tumor cell ratio was 20:1). Cultures were pulsed with 1 microcurie of ^{3}H-thymidine (^{3}H-TdR) (specific activity, 20 curies/mmol; CEA, France) for 6 hours before harvesting. Tumor cells were collected on filters using a multiple harvesting device and counted for ^{3}H-TdR incorporation in a liquid scintillation counter.

Lymphocyte response to mitogens.—Spleens from four mice in each group were pooled and prepared as single cell suspensions. Spleen cells were fractionated by nylon wool filtration according to the method of Julius et al (11). Unfractionated spleen cells or nylon-non-adherent spleen cells (5×10^5) in 0.2 ml of RPMI-1640 culture medium, supplemented with 5% heat-inactivated mule serum and antibiotics, were seeded into each well of a culture microplate. Mitogens were added at the dose found to be optimal in preliminary experiments: phytohemagglutinating (PHA) (Wellcome Research Laboratories), 1 μg/ml; dextran sulfate (DS) (molecular weight, 5×10^5; Gibco), 250 μg/ml. Cultures were incubated for 54 hours at 37°C in an atmosphere of 5% CO_2 and 95% air, with the addition of 1 microcurie of ^{3}H-TdR to each well for the final 6 hours of the culture period. Cells were harvested and prepared for radioactivity counting as described above.

Assay for detecting suppressor cells.—Normal spleen cells (5×10^5) were cultivated in the presence of 3×10^5 unfractionated, nylon-non-adherent, or plastic-adherent spleen cells from adjuvant-treated mice; cultures were stimulated with PHA or DS and processed as described above.

Antibody response.—Control and adjuvant-treated mice (eight mice per group) were immunized ip with various doses of sheep red blood cells (SRBC) or with one of the following trinitrophenyl (TNP) carrier conjugates in saline: 300 μg of TNP-hemocyanin (KLH) or 10 μg of TNP-polymerized flagellin (POL). The antibody-forming cell response of these animals was measured at their respective peaks (3, 4, and 6 days after TNP-POL, SRBC, and TNP-KLH injections, respectively). Plaque-forming cells (PFC) in the spleen were enumerated by using a modification of Cunningham's hemolytic plaque assay with SRBC or TNP-coated SRBC as indicator cells.

Delayed-type hypersensitivity reaction.—Control or adjuvant-treated mice (ten animals per group) were sensitized by application of 0.1 ml of a 3% oxazolone solution in absolute alcohol to the shaven abdomen skin. Seven days after sensitization, 10 μl of a 1% oxazolone solution in olive oil was applied to both sides of each ear. Ear thickness was measured just before and 24 hours after the challenge.

Antibody-dependent cell-mediated cytotoxicity (ADCC).—Spleen cell suspensions were adjusted to a final concentration of 10^7 cells/ml of RPMI-1640 medium supplemented with 10% FCS. Chicken erythrocytes (CRBC) (5×10^6) were incubated for 1 hour at 37°C in 0.5 ml of phosphate buffer, pH 7.4, containing 500 microcuries of $Na_2{}^{51}CrO_4$ (NEN Chemicals, 200–500 curies/g) and then washed three times before use at a concentration of 10^5 cells/ml. The test was performed in round-bottom microtiter plates (Linbro), each well containing 0.1 ml of the spleen suspension (10^7 cells/ml), 0.1 ml of ^{51}Cr-labeled CRBC suspension (10^5 cells/ml), and 20 μl of a different dilution of anti-CRBC antiserum raised in rabbits (twofold dilution varying from 1:8 to 1:128,000). At the end of a 16-hour incubation at 37°C (95% air, 5% CO_2), the radioactivity of the supernatant was counted in a gamma counter.

RESULTS

Effects of BCG and Levamisole on the Immune Responses of Young Adult Mice

Two-month-old mice were given a single iv injection of 0.05–5 mg of BCG or levamisole at a dose of 25 or 75 μg. The effects on various humoral and cell-mediated immune responses were tested 14 days after BCG inoculation and 7 or 14 days after levamisole injection.

Effect on Macrophage Activation

As shown in table 1, peritoneal macrophages were not cytostatic in vitro for leukemic cells 14 days after the injection of 0.05 mg of BCG. The tumoricidal activity was detected after 0.2 mg of BCG and was maximal after 1 or 5 mg. In contrast, levamisole was unable to induce macrophage cytostatic activity whatever the dose or the time of administration.

TABLE 1.—Macrophage activation measured by cytostatic activity for tumor cells of mice treated with BCG or levamisole

Adjuvant treatment	Iv dose	Time interval* Day 7	Time interval* Day 14
BCG	0.05 mg		0
	0.2 mg		47
	1 mg		90
	5 mg		99
Levamisole	25 μg	11	+11 (stimulating effect)
	75 μg	0	7

* % of inhibition of TdR incorporation into tumor cells compared to controls =

$$100 - \left[\frac{\text{mean cpm in adjuvant-treated macrophage cultures}}{\text{mean cpm in normal macrophage cultures}} \times 100\right].$$

Effects on Antibody Responses

BCG- or levamisole-treated mice were immunized with 10^9 SRBC ip and the numbers of direct PFC per spleen were determined 4 days later (table 2). Treatment with 0.05 mg of BCG did not result in the enhancement of the PFC response as compared with the untreated animals. A slight but significant increase of the PFC number per spleen was observed after 0.2 mg of BCG. This potentiating effect was more marked after 1 mg of BCG. Varying the dose of levamisole and the time of its injection with respect to antigen administration did not modify the anti-SRBC-PFC response.

In order to define the site of action of BCG in the antibody response, animals were immunized against TNP-haptenic groups coupled on thymus-dependent (KLH) or thymus-independent (POL) carriers. Results are presented in table 3. No modification of both thymus-dependent and thymus-independent antibody responses was observed when mice were treated with 0.2 mg of BCG. A threefold increase in the number of anti-TNP-PFC per spleen was detected in mice given 1 mg of BCG and immunized against TNP-KLH or TNP-POL. Five milligrams of BCG exerted a similar stimulatory effect on the PFC response to TNP-KLH but was less efficient in potentiating the response to TNP-POL for which only a twofold increase in the number of PFC per spleen was observed.

TABLE 2.—Antibody response to SRBC in young adult mice treated with BCG or levamisole

Adjuvant treatment	Iv dose	Time interval* Day 7	Time interval* Day 14
BCG	—	—	222,750 ± 12,856
	0.05 mg	—	168,333 ± 15,418
	0.2 mg	—	281,668 ± 20,256†
	1 mg	—	389,430 ± 25,810†
Levamisole	—	23,714 ± 5000	—
	25 μg	21,500 ± 14,500	27,000 ± 3000
	75 μg	18,000 ± 3500	24,500 ± 6500

* Mean No. of PFC to SRBC ± SE/spleen.
† Significantly different from responses of untreated mice.

TABLE 3.—Effect of different doses of BCG on PFC response to thymus-dependent (TNP-KLH) and thymus-independent (TNP-POL) antigens

Antigens	Dose (mg) of BCG* None	0.2	1	5
TNP-POL	13,080 ± 1690	16,920 ± 2718	42,120† ± 4139	28,500† ± 5730
TNP-KLH	3825 ± 595	3550 ± 1061	12,625† ± 1510	12,200† ± 795

* Results are expressed as mean No. of PFC ± SEM/spleen.
† Significantly different from responses of untreated mice.

Effects on In Vitro Lymphocyte Responses to Mitogens

Spleen cells from normal mice and from BCG- or levamisole-treated mice were tested for their ability to respond to a T-cell mitogen (PHA) and a B-cell mitogen (DS), either unfractionated or after filtration through a nylon wool column. The mitogen response of adjuvant-treated spleen cells is expressed as the percentage of mitogen-induced ^{3}H-TdR incorporation into spleen cells from untreated mice.

Increasing doses of BCG from 0.2 to 5 mg resulted in a progressive depression of the PHA response of unfractionated spleen cells whereas concomitantly the response to the B-cell mitogen was increased (fig 1A). This defective response to PHA was completely restored after elimination of nylon-adherent cells, ie, B lymphocytes and macrophages (11), when the animals were treated with 0.2 or 1 mg of BCG. This restoration was only partial when spleen cells were obtained from mice treated with 5 mg of BCG (fig 1A). Both T- and B-cell mitogen responses of whole spleen cells from levamisole-treated mice were augmented on Day 7 but returned to normal values on Day 14 after adjuvant administration. The nylon-non-adherent (T-cell-enriched) population exhibited a normal reactivity to PHA regardless of the time of levamisole administration (fig 1B).

The following experiments were performed in order to test whether the depressive response to PHA of spleen cells from mice given BCG at a dose $\geq$ 0.2 mg may be due to the induction of nonspecific suppressor cells. Unfractionated normal spleen cells were stimulated with PHA and DS in the presence

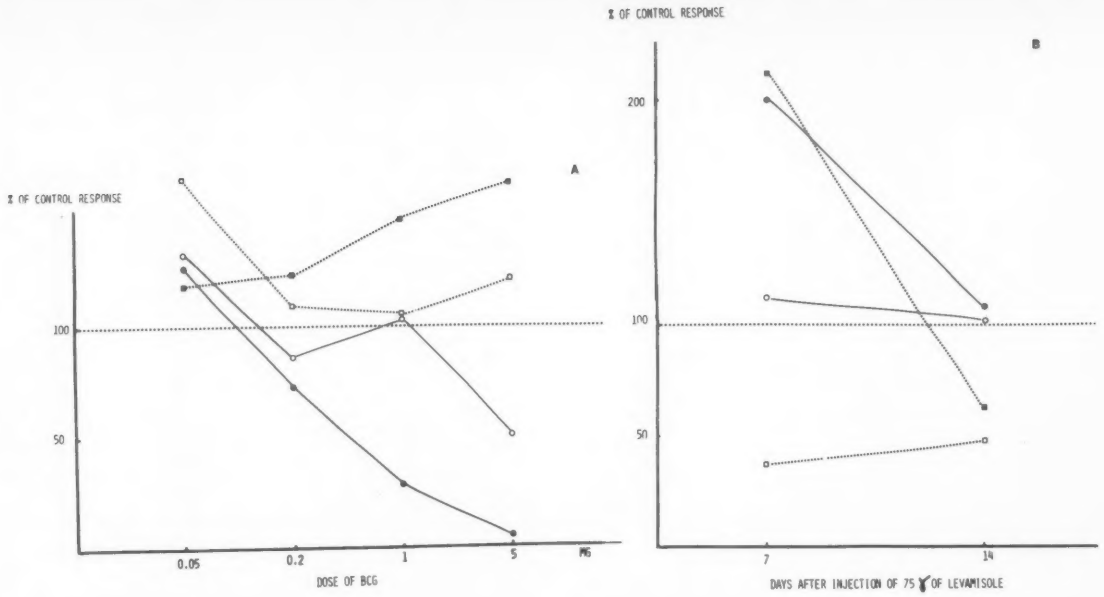

FIGURE 1.—Mitogen responsiveness to PHA (———) or DS (· · · · ·) of 5×10^5 unfractionated (● ■) or 5×10^5 nylon-non-adherent spleen cells (○ □) from mice treated with various doses of BCG (A) or levamisole (B). Results are expressed as follows:

$$\% \text{ of control response} = \frac{\text{mean cpm in the spleens of treated mice}}{\text{mean cpm in the spleens of normal mice}} \times 100.$$

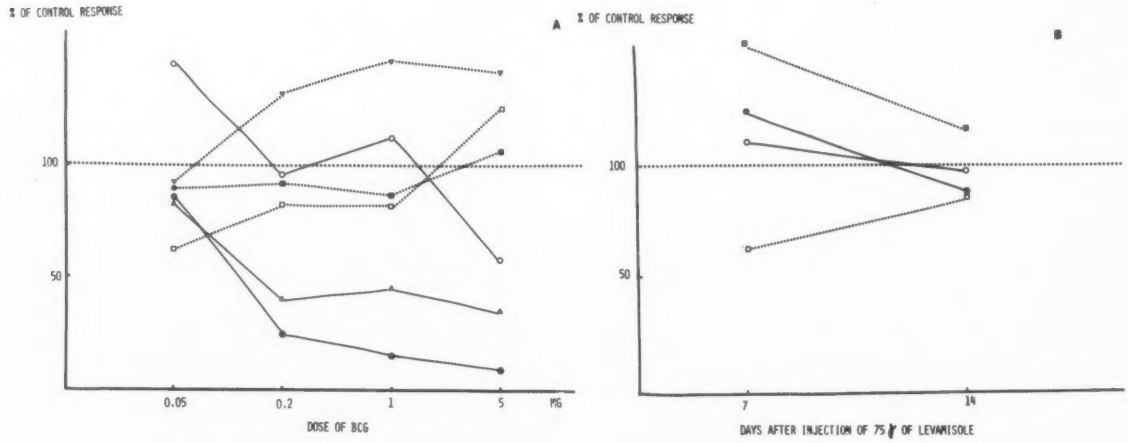

FIGURE 2.—Mitogen responsiveness to PHA (———) or DS (· · · · ·) of 5×10^5 normal spleen cells cultivated in the presence of 3×10^5 unfractionated (● ■) or 3×10^5 nylon-non-adherent (○ □) or 10^5 adherent (△) spleen cells from mice treated with various doses of BCG (A) or levamisole (B).

$$\% \text{ of control response} = \frac{\text{mean cpm in the spleens of treated mice}}{\text{mean cpm in the spleens of normal mice}} \times 100.$$

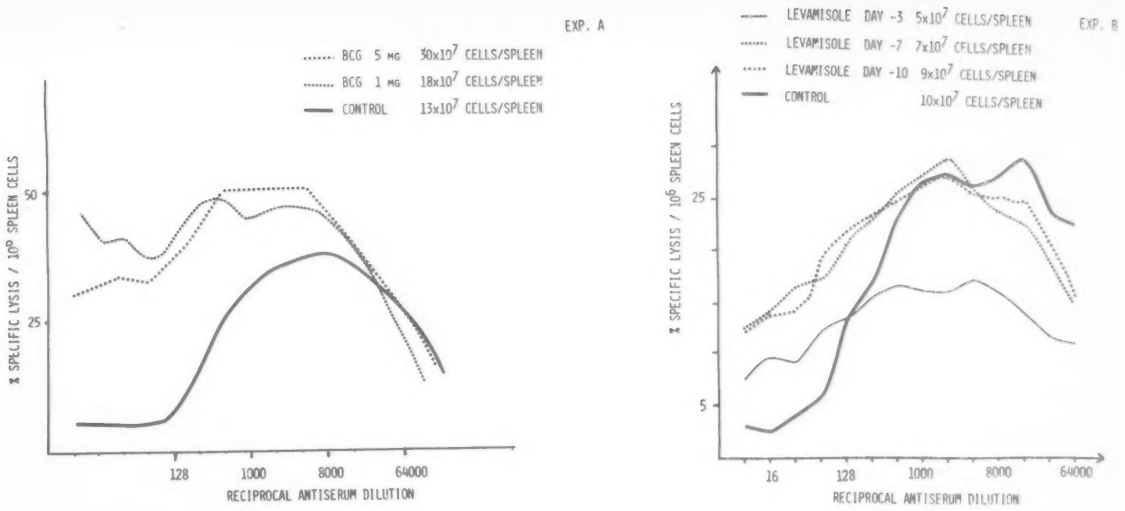

FIGURE 3.—Effect of BCG and levamisole on ADCC for 10^6 spleen cells. Each sample was assayed in triplicate and results are expressed as follows:

$$\% \text{ specific } {}^{51}\text{Cr release} = \frac{\text{test release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100.$$

of different spleen cell subpopulations from BCG-treated mice. Their proliferative responses were expressed as the percentage of the response observed when they were admixed with the respective subpopulation of normal spleen cells. As shown in figure 2A, the PHA response of normal spleen cells was nearly completely abolished by the addition of unfractionated spleen cells from BCG-treated mice. Nylon-non-adherent cells from mice treated with 0.2 or 1 mg of BCG were ineffective in decreasing the PHA response of normal spleen whereas the same population coming from mice treated with 5 mg of BCG still induced a 40% depression of this response (fig 2B). When normal spleen cells were cultivated with additional plastic-adherent spleen cells from BCG-treated mice, a depressed PHA response was observed and the inhibitory effect increased with the dose of BCG. The response to DS of normal spleen cells was either not affected or slightly enhanced by the addition of BCG-treated spleen cells.

These experiments strongly suggested that macrophage-like suppressor cells were responsible for the depression of the spleen cell PHA responsiveness observed after administration of BCG at a dose ≥ 0.2 mg and that additional suppressor cells, probably T cells, were also induced when 5 mg of BCG was given.

Nonspecific suppressor cells could not be evidenced after levamisole administration since unfractionated and nylon-non-adherent spleen cells from adjuvant-treated mice were ineffective in decreasing the PHA response of normal spleen cells (fig 2B).

Delayed-Type Hypersensitivity Reaction

The augmentation of ear thickness, observed 24 hours after application of the challenge dose of oxazolone 7 days after sensitization, was significantly more marked in mice given only 0.05 mg of BCG. This stimulatory effect was augmented with the dose of BCG. A twofold increase in ear thickness was observed after 1 mg of BCG when compared to untreated mice (table 4).

Effect on ADCC

Spleen cells from normal mice and from BCG- or levamisole-treated mice were compared for their ability to kill CRBC in the presence of rabbit antibody at various dilutions. The data shown in figure 3 were obtained with a single effector to target cell ratio of 100:1.

Spleen cells from BCG-treated mice displayed a significantly higher cytotoxic activity on antibody-coated CRBC than normal spleen cells for a final antiserum dilution of 1:80 to 1:80,000. Moreover, a strong cytotoxicity could be demonstrated with BCG-treated spleen cells at high antiserum concentrations (from 1/80 to 1/2560) when normal spleen cells were inactive. This suggested that different cell populations were implicated in ADCC in normal and BCG-treated spleen cells.

TABLE 4.—Effect of BCG and levamisole on delayed-type hypersensitivity to oxazolone in adult young mice

Adjuvant treatment before sensitization with oxazolone on Day 0	Iv dose	Ear thickness (1/100 mm)			
		Before challenge	24 hrs after challenge	Increment of ear thickness (1/100 mm)	
BCG, Day –14	0	31.60 ± 2.39	47.63 ± 2.39	16.03 ± 3.34	
	0.05 mg	30.70 ± 1.82	53.20 ± 3.01	22.50 ± 2.45 $P = 0.01$	
	0.2 mg	31.83 ± 1.26	55.00 ± 2.29	23.17 ± 2.36 $P = 0.02$	
	1 mg	32.00 ± 1.17	60.30 ± 3.85	28.30 ± 3.73 $P < 0.01$	
Levamisole					
Day –7	0	33.90 ± 1.95	52.90 ± 5.00	19	
	25 μg	34.40 ± 2.20	52.70 ± 5.70	18.3	NS*
	75 μg	33.50 ± 2.10	53.60 ± 4.60	20.1	NS
Day –14	0	29.40 ± 1.25	49.10 ± 3.65	19.7	
	25 μg	28.40 ± 1.90	48.50 ± 7.60	20.1	NS
	75 μg	29.80 ± 1.09	49.20 ± 3.05	19.4	NS

*NS = not significant.

TABLE 5.—PFC response to various doses of SRBC in young and aged mice

Age of mice	Mean No. of PFC/spleen ± SE in response to:		
	10^5 SRBC	10^7 SRBC	10^9 SRBC
3 mos	150 ± 80	93,000 ± 31,000	206,000 ± 40,000
12 mos	50 ± 30	21,200 ± 14,000	123,000 ± 54,000

Spleen cells from mice given 75 μg of levamisole 7 or 10 days before testing were able to kill antibody-coated CRBC more efficiently than normal spleen cells in the presence of high concentrations of antibodies. At final concentrations of antiserum > 1/2500, no significant difference between ADCC activity of normal or levamisole-treated spleen cells was observed. When levamisole was injected 3 days before testing, spleen cells displayed significantly lower cytotoxic activity than normal spleen cells at the same antiserum concentrations.

Effects of BCG and Levamisole in Aged Mice

Effect of Aging on Immune Responses

Three- and 12-month-old mice were compared for their response to SRBC in vivo and for their spleen cell reactivity to mitogens in vitro. The PFC response rates after immunization with 10^5, 10^7, or 10^9 SRBC were reduced 67%, 78%, and 40%, respectively, in 12-month-old mice when compared to response rates in 3-month-old mice (table 5). The less marked depression observed after injection of a high dose of SRBC may be explained by the fact that in this condition the antibody response is partly thymus-independent.

A 40% reduction of the response of whole spleen cells to T-cell mitogens (PHA and concanavalin A) was observed in 12-month-old mice, the response to a B-cell mitogen being less affected (table 6). This defective response may result from a decrease in the number of mitogen reactive cells or the induction of natural nonspecific suppressor cells which were not T cells. Indeed, after nylon filtration, the T-cell-enriched population exhibited the same proliferative response to PHA and concanavalin A as the 3-month-old spleen cells (table 6).

The capacity for macrophages to be activated by BCG and thus to exert cytostatic effects on tumor cells in vitro remained unaffected by aging (results not shown).

Effects of BCG and Levamisole Treatments on Immune Responses

Twelve-month-old mice were given weekly ip injections of 1 mg of BCG or 75 μg of levamisole over a period of 6 months. The animals were tested for macrophage activation, antibody response to SRBC, and spleen cell responsiveness to mitogens 2 weeks after the last injection, and their responses were compared to those of 18-month-old mice either untreated or given a single injection of BCG or levamisole 14 days before testing.

TABLE 6.—Mitogen responsiveness of spleen cells of young and aged mice

Population of spleen cells	Mitogen	Mice aged— 3 mos	12 mos
5×10^5 unfractionated spleen cells	—	312†	625
	PHA	28,455	17,856
	Con A*	36,142	25,842
	DS	18,545	14,071
5×10^5 nylon-non-adherent spleen cells	—	328	103
	PHA	38,577	41,355
	Con A	29,015	28,662
	DS	1065	530

*Con A = concanavalin A.
†Mean cpm in triplicate cultures.

TABLE 7.—Macrophage activation measured by cytostatic activity for tumor cells and antibody response to SRBC in 18-mo-old mice treated with BCG or levamisole over a period of 6 mos

Adjuvant treatment	Controls	BCG 1 mg × 1	BCG 1 mg weekly	Levamisole 75 μg × 1	Levamisole 75 μg weekly
% of inhibition of TdR incorporation into tumor cells compared to controls*	0	91	99	+11 (stimulating effect)	NT†
Mean No. of PFC to SRBC ± SE/ spleen	18,333 ± 3400	26,608 ± 3361	3464 ± 2951	28,312 ± 7245	47,108 ± 12,945

*% of inhibition $= 100 - \left[\frac{\text{mean cpm in adjuvant-treated macrophage cultures}}{\text{mean cpm in normal macrophage cultures}} \times 100\right]$.
†NT = not tested.

The results presented in table 7 show that peritoneal macrophages from aged mice given one or more injections of BCG were highly cytostatic for tumor cells. In contrast, macrophages from mice treated with one injection of levamisole did not display cytostatic effect.

The PFC response to 10^9 SRBC was markedly depressed in 18-month-old mice (18,000 versus 93,000 in 3-month-old mice). The continuous administration of BCG over a period of 6 months resulted in drastic aggravation of this depression, the number of PFC per spleen being only 15% of the number observed in untreated mice. In contrast, multiple injections of levamisole were followed by a threefold increase in the number of PFC per spleen and thus this treatment partially restored the antibody response of age-immunodepressed mice. A single injection of BCG and of levamisole slightly but significantly enhanced (40%) the PFC response (table 7).

Table 8 shows that the response to PHA of unfractionated spleen cells from 18-month-old mice was depressed by 50% after repeated treatment with BCG or levamisole. This depressive effect was less pronounced (30% reduction) after a single injection of BCG. A restoration of the response was observed after filtration through nylon wool columns except in mice receiving multiple BCG treatments. Cocultivation experiments showed that suppressor cells were present in the spleen of BCG-treated mice and of mice receiving multiple levamisole treatments since unfractionated spleen cells from these animals significantly inhibited the PHA response of normal spleen cells. These suppressor cells appeared to be only macrophage-like cells in the case of single BCG and multiple levamisole injection treatments since nylon-non-adherent cells were not able to suppress the response of normal spleen cells. Moreover, in the case of continuous BCG administration, in addition to suppressor macrophages, another population, probably T lymphocytes, was induced. Nylon-non-adherent spleen cells from these animals significantly depressed the PHA response of normal spleen cells.

The response to DS was not modified after levamisole treatment and was significantly enhanced after single or continuous BCG administration.

TABLE 8.—Mitogen responsiveness of 18-mo-old mice treated with BCG or levamisole

Population of spleen cells	Mitogen	Controls	Spleen cells from mice treated with—			
			BCG		Levamisole	
			1 mg × 1	1 mg weekly	75 μg × 1	75 μg weekly
5×10^5 unfractionated spleen cells	—	157*	328	1701	375	115
	PHA	5295	3593	2612	6543	2295
	DS	434	1006	1405	440	610
5×10^5 nylon-non-adherent spleen cells	—	98	415	1633	141	312
	PHA	11,650	9663	4702	9950	10,297
	DS	616	833	1145	317	997
5×10^5 normal spleen cells cultivated with—						
3×10^5 unfractionated spleen cells	—	276	696	1140	572	860
	PHA	8515	3112	4235	12,189	5687
	DS	3487	3400	2311	3188	4632
3×10^5 nylon-non-adherent spleen cells	—	357	460	1424	303	528
	PHA	11,523	12,886	7359	9445	12,030
	DS	739	2572	2343	640	989

* Mean cpm in triplicate cultures.

Effect of BCG and Levamisole Treatment on Spontaneous Tumor Incidence

The survival of the animals was recorded after stopping the continuous treatment with BCG or levamisole. All BCG-treated mice died within 3 months after treatment was discontinued, with a median survival time of 584 days. In contrast, 50% of the untreated mice and 66% of the levamisole-treated mice were alive at the age of 24 months (fig 4). At this time, the animals were killed and examined for the presence of spontaneous tumors. The tumor incidence appeared to be reduced in the group of mice treated with levamisole: 22% compared to 83% in the untreated mice.

DISCUSSION

The data presented in this paper show that in young adult mice, a single injection of BCG was able to potentiate various immune responses while levamisole was ineffective regardless of the dose and time of administration. Different results were obtained when these agents were injected repeatedly to age-immunodepressed mice. Continuous treatment with levamisole resulted in a partial restoration of the antibody response which was depressed by aging whereas continuously administered BCG strongly aggravated the immunodepressive state. Simultaneously, nonspecific macrophage-like suppressor cells were induced in the spleens of both levamisole- and BCG-treated mice and additional T-suppressor cells were also detected only in the case of BCG treatment.

We have already described some effects of BCG on immune responses in young adult mice when they were injected with a moderate dose (1 mg) (1). In the studies presented here, we observed that the minimal dose of BCG which can modify immune reactivity was 0.2 mg except for a delayed-type hypersensitivity reaction which was potentiated after only 0.05 mg of BCG. Concomitantly to the increase of humoral responses against thymus-dependent and thymus-independent antigens and of delayed-type hypersensitivity, a profound depression of lymphocyte reactivity to T-cell mitogens, in vitro, was observed. This effect was demonstrated to depend upon the presence of nonspecific suppressor cells which, therefore, did not seem to exert some inhibitory effect in vivo. Similar observations were done when BCG was repeatedly administered to young adult mice (manuscript in preparation). Different results were observed when BCG was continually injected in aged mice. Under these conditions, the detection of suppressor cells in vitro was accompanied by an important decrease of the antibody response to SRBC in vivo. It may be suggested that the regulatory role of BCG-induced suppressor cells is more easily detectable in immunodepressed mice. Inhibition of a graft-versus-host reaction was reported in highly irradiated mice given a mixture of normal and BCG-treated lymphoid cells (12). This hypothesis could help explain why tumor enhancement is sometimes observed during BCG immunotherapy (2,13,14) since immunologic impairment is generally associated with tumor development. Several reports now demonstrate that BCG stimulates the appearance of suppressor cells. These cells were evidenced in vitro by the inhibition of either lymphocyte proliferative response to mitogens (15,16) or generation of cytolytic T lymphocytes

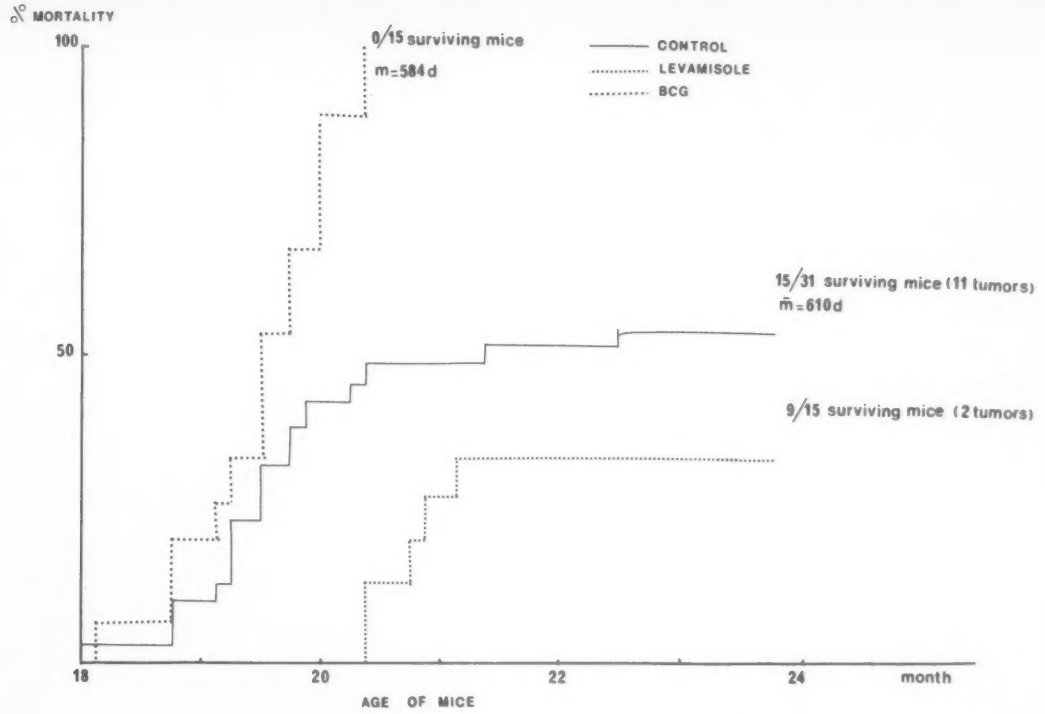

FIGURE 4.—% of mortality of 18-mo-old mice either untreated or treated repeatedly with BCG or levamisole. The surviving mice were killed at 24 mos of age and the number of tumor-bearing mice was recorded.

(17,18) and were identified as macrophages. In this study, we have presented evidence that after high doses of BCG, an additional population of suppressor cells developed which presented T-cell characteristics: non-adherence to nylon and sensitivity to Thy-1 antiserum (unpublished data).

We have demonstrated that BCG increased the cytotoxic activity of spleen cells for antibody-coated CRBC. This effect was observed over a large range of antibody concentrations, suggesting that several types of effector cells, differing in the density of their Fc receptors or the affinity or specificity of these receptors, were present in the spleen of BCG-treated mice. These results are consistent with previous observations that BCG-induced tumor regression mainly depended upon an increase of ADCC activity (19).

Little information is available about the in vivo effects of levamisole administration in immunocompetent animals. Enhancement of antibacterial immunity (20) and increased production of antibody-forming cells (21) have been described but generally negative results have been reported (22). Levamisole, when injected in young adult mice, was ineffective in increasing antibody response against SRBC, delayed-type hypersensitivity reaction, and lymphocyte response to T- and B-cell mitogens. It did not induce macrophage activation or the appearance of suppressor cells. ADCC of spleen cells was either slightly enhanced or depressed according to the time of levamisole injection.

The repeated administration of levamisole to 12-month-old mice resulted in a restoration of the antibody response to SRBC which was depressed by aging. In contrast, continuous treatment with BCG aggravated this immunodepressive state. Macrophage-like suppressor cells were detected in the spleens of levamisole- and BCG-treated mice but in the second instance, T-suppressor cells were also evidenced suggesting that these cells were responsible for the defective PFC response in BCG-treated mice.

Levamisole has been shown to influence the primary growth and dissemination of various experimental tumors (8–10). We have observed that spontaneous tumor incidence is significantly reduced in mice continuously treated with levamisole. The mechanism of this protective effect is not eluci-

dated. Levamisole may act in increasing naturally cytotoxic (NK) activity as already demonstrated for BCG (23). Experiments are in progress to define the target cells, T lymphocytes, K and NK cells, and macrophages of levamisole action in age-immunodepressed mice.

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Effects of Combined Immunotherapy With Levamisole and Bacillus Calmette-Guérin on Immunocompetence of Patients With Squamous Cell Carcinoma of the Cervix, Head and Neck, and Lung Undergoing Radiation Therapy [1]

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SUMMARY

Patients with squamous cell carcinoma of the head and neck, squamous cell carcinoma of the cervix, and lung carcinoma were treated with radiation therapy (RT) prior to being randomly assigned either to a group receiving no further treatment or to a group treated with combined adjuvant immunotherapy (bacillus Calmette-Guérin and levamisole). A battery of in vitro immunologic evaluations in addition to skin tests was used to evaluate these patients prior to RT, immediately following RT, and at regular intervals thereafter. Mean percentages and levels of circulating T lymphocytes were significantly lower in all three types of patients prior to RT than in normal healthy controls. B-lymphocyte percentages and levels, however, were not significantly different from controls except for lower B-cell levels in the lung group. Following completion of RT, circulating levels of both T and B lymphocytes were significantly lower than pretreatment values although the percentages were not significantly changed. Mitogenic responses of patient lymphocytes to both phytohemagglutinin and pokeweed mitogen were significantly lower prior to RT than were healthy control responses. A further depression of blastogenesis following RT was statistically significant. Preliminary data at intervals following RT indicate a gradual recovery of depressed immune parameters (T- and B-lymphocyte levels and mitogenic responses) both in patients treated with adjuvant immunotherapy and in those receiving no further treatment. Although not statistically significant in preliminary data, there is a suggestion that recovery of these immune parameters is slower in the group receiving immunotherapy. Plasma sialic acid levels were elevated in patients when compared to healthy controls and remained elevated throughout the study with little fluctuation. Lymphocyte cytotoxic activity against tumor target cells was variably affected by RT, but was generally increased at 8 weeks following RT when compared to previous values.

[Cancer Treat Rep 62:1651–1661, 1978]

Impaired immune parameters in vitro in most patients with squamous cell carcinoma at the time of diagnosis (1–3), with further depression by therapeutic modalities, prompted us to apply bacillus Calmette-Guérin (BCG) and levamisole in randomized trials in an attempt to improve patient immunity following completion of radiation therapy (RT).

MATERIALS AND METHODS

A total of 125 patients were studied, 43 with carcinoma of the lung (disease localized to the thorax), 25 with squamous cell carcinoma of the cervix (stages Ib, II, and III), and 57 with squamous cell carcinoma of the head and neck (stages II, III, and IV limited to the neck). Of these patients, a

[1]Supported by contract NCI-CB-64004-31 from the Division of Cancer Cause and Prevention, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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total of 98 have been followed with immunologic evaluations for sufficient time intervals to be analyzed. Patients were evaluated immunologically both before and after the full course of RT and were then randomized to receive either immunotherapy or no further treatment. Immunotherapy consisted of levamisole (150 mg/day orally, 2 days/week) and BCG, Tice strain, 3×10^8 viable units/vial, applied intradermally by the multiple puncture disc method (one vial per application). Patients with head and neck and lung cancer received vaccinations in alternating arms while patients with cervical cancer received BCG in alternating thighs. All immunotherapy patients received BCG every 2 weeks for the first 2 months and then once a month for the next 10 months.

Immunologic evaluations were repeated on patients in both the immunotherapy and no-immunotherapy groups at intervals of 8, 16, 28, 40, and 52 weeks following the completion of RT. The response of the immune system was monitored using a battery of skin tests (mumps, purified protein derivative [PPD], *Candida*, streptokinase, and dinitrochlorobenzene [DNCB]), circulating T- and B-lymphocyte levels (1,4), phytohemagglutinin (PHA)- and pokeweed mitogen-induced lymphocyte blastogenesis in vitro (5), lymphocyte cytotoxicity to allogeneic tumor cell lines (6), and plasma sialic acid levels (7). Samples from 85 normal healthy individuals were assayed concurrently as controls.

RESULTS

T Lymphocytes

Table 1 shows mean percentages and levels of circulating T lymphocytes in patients with all three categories of carcinoma evaluated before RT, at the completion of RT, and at 8, 16, 28, and 40 weeks following RT compared to normal control values. Patients are divided into immunotherapy and no-immunotherapy groups at the latter intervals. At the time of diagnosis the mean percentages of T lymphocytes were significantly reduced below control values of 61.6% to mean levels of 53.4% for those with head and neck cancer, 55.3% for those with lung cancer, and 54.3% for those with cervical cancer. At the end of RT little change was noted in these values. Although at several intervals after the completion of RT there was a tendency for these values to return to normal levels, very little variation in mean T-cell percentages was observed.

The mean levels of circulating T lymphocytes at the time of diagnosis for head and neck, lung, and cervical cancer patients were 1082, 1071, and 989 cells/mm^3 respectively, which were all significantly lower ($P < 0.05$) than that of the 85 healthy controls (1277 cells/mm^3). At the completion of RT these values were further reduced to approximately 35%–40% of their pretreatment values. At various intervals following RT the levels of T lymphocytes tended to increase toward pretreatment values and, of interest, the increase is somewhat more marked for those groups to which no immunotherapy was administered. Because this study is in progress and therefore relatively small numbers of patients have been evaluated at intervals > 16 weeks, a statistical analysis comparing these results for the immunotherapy versus no-immunotherapy group was appropriate only for early data. T-lymphocyte percentages and levels in patients who were evaluated at all intervals through 16 weeks were examined using analyses of variance with a repeated measures design. Group means are presented in figure 1 for the head and neck group and in figure 2 for the lung group. Both percentages and levels of T cells are lower than the controls in the head and neck patient groups before treatment. On the last day of RT, T-cell levels showed a striking and significant decrease when compared to pretreatment values, although T-cell percentages were not significantly changed. T-cell percentages in the patients remain relatively stable through subsequent evaluation intervals, while T-cell levels show a gradual increase in both the immunotherapy and no-immunotherapy groups. Although there appears to be a trend toward more rapid recovery in the no-immunotherapy group, the difference is not statistically significant at this point. The lung patient groups show a response pattern similar to the head and neck groups. Once again, T-cell percentages remain relatively stable, although initially lower than normal controls. T-cell levels, however, show a dramatic and significant decrease at the end of RT with a trend toward recovery 8 weeks later. Recovery is again slightly but not significantly better in the group receiving no immunotherapy. Due to the small number of patients with cervical cancer evaluated at these intervals, analysis of variance was not performed in this group.

B Lymphocytes

Percentages of B lymphocytes prior to RT did not differ significantly ($P < 0.05$) from healthy control values in any of the patient groups. Paired analysis of pre-RT and post-RT percentages in patients also showed no significant differences. Levels of circulating B cells were significantly depressed below control only in the lung patient group prior to RT. At the completion of RT, however, paired analysis revealed that in all three populations of patients

TABLE 1.—% circulating T lymphocytes and T-cell levels*

		Carcinoma								
		Head and neck			Lung			Cervix		
Time	Immunotherapy	No. of patients	% T cells	T-cell level	No. of patients	% T cells	T-cell level	No. of patients	% T cells	T-cell level
Before RT		57	53.4 ± 12.8†	1081.8 ± 466.5†	40	55.3 ± 14.0†	1071.1 ± 588.5†	24	54.3 ± 14.1†	989.4 ± 485.0†
Last day of RT		36	55.8 ± 14.9	471.6 ± 290.7‡	24	58.3 ± 15.1	392.2 ± 178.0	14	55.4 ± 16.8	328.3 ± 213.1‡
8 wks after RT	No immunotherapy	15	54.2 ± 13.7	719.6 ± 182.0	8	64.6 ± 13.1	710.3 ± 258.3	6	64.0 ± 12.3	631.0 ± 123.7
	Immunotherapy	13	54.2 ± 17.4	508.1 ± 210.5	7	54.4 ± 22.2	485.4 ± 335.2	6	57.0 ± 7.7	592.7 ± 257.2
16 wks after RT	No immunotherapy	11	61.5 ± 13.2	884.0 ± 368.4	7	56.6 ± 13.2	661.9 ± 358.0	6	57.0 ± 8.6	500.3 ± 100.9
	Immunotherapy	10	52.9 ± 16.3	475.1 ± 213.6	4	61.3 ± 13.4	694.8 ± 273.4	6	53.7 ± 13.4	601.8 ± 237.8
28 wks after RT	No immunotherapy	5	61.6 ± 15.7	1086.8 ± 477.2	4	49.0 ± 10.9	599.3 ± 128.5	2	50 ± 2.8	355.0 ± 277.2
	Immunotherapy	8	54.1 ± 23.4	508.6 ± 227.1	1	77.0	377.0	4	64.0 ± 20.3	955.0 ± 439.0
40 wks after RT	No immunotherapy	3	46.0 ± 10.6	723.0 ± 151.6				2	63.0 ± 5.7	523.0 ± 31.1
	Immunotherapy	3	56.7 ± 6.0	631.0 ± 130.2				4	46.5 ± 35.6	525.0 ± 345.7

*Control T-cell level = 1277 cells/mm³ (85 controls); control % T cells = 61.6 ± 10 (85 controls).
†Significantly different from control at $P < 0.05$.
‡Significantly different from before RT at $P < 0.05$.

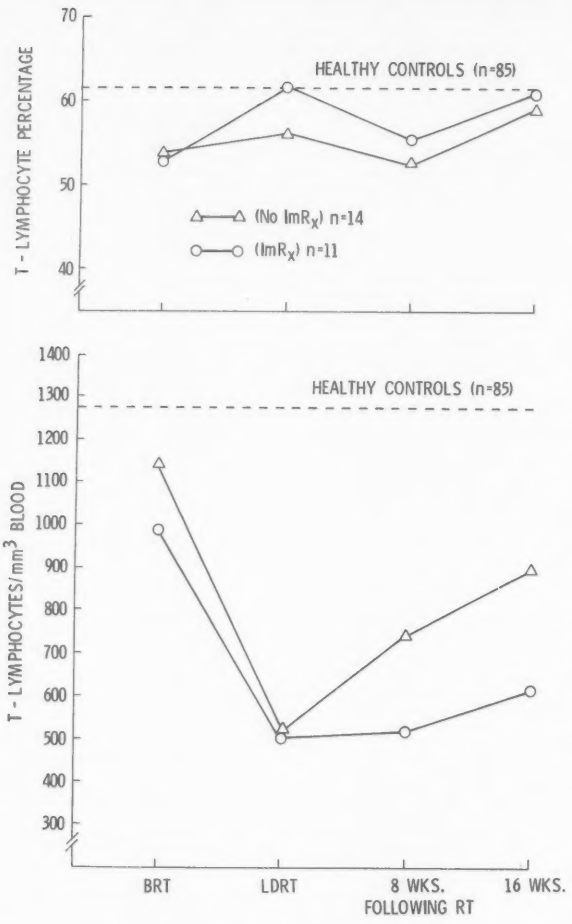

FIGURE 1.—T-cell evaluations in head and neck carcinoma patients.

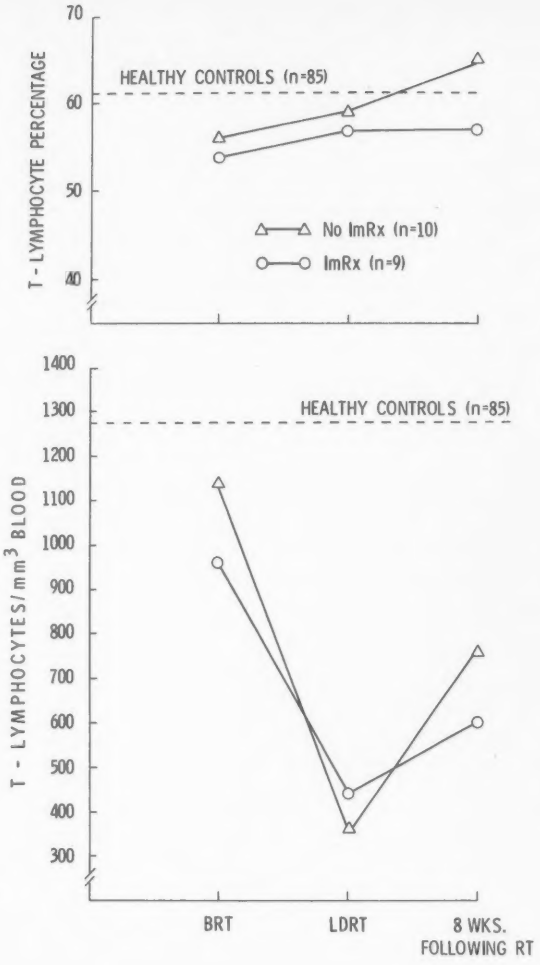

FIGURE 2.—T-cell evaluations in lung carcinoma patients.

the levels were significantly depressed below pre-RT levels. Eight weeks following the completion of RT these values in all three groups of patients had returned approximately half-way toward pre-RT values. In general, the improvement was somewhat more marked for those individuals who did not receive immunotherapy compared to those receiving the immunotherapy protocol, with the possible exception of those with cervical cancer, who were observed beyond 8 weeks. The numbers of observations from this group are quite small, however, and require confirmation. Insufficient data were available for a statistical comparison of the immunotherapy versus no-immunotherapy groups.

Lymphocyte Blastogenesis

PHA stimulation of lymphocyte blastogenesis in vitro is presented in table 2. The results are expressed as cpm. At the time of diagnosis, blastogenesis was significantly reduced below the mean control values of 160,000 cpm to 113,000, 74,000, and 87,000 cpm for patients with head and neck, lung, and cervical cancer respectively. Further statistically significant reductions (paired analysis) below these low levels were found following RT (to 67,000, 40,000, and 26,000 cpm respectively). At the various intervals after the RT the values in those patients with head and neck and cervical cancer tended

TABLE 2.—PHA- and pokeweed mitogen-stimulated lymphocyte blastogenesis in vitro*

		Carcinoma								
		Head and neck			Lung			Cervix		
Time	Immunotherapy	No. of patients	PHA (cpm × 10^{-4})	Pokeweed mitogen (cpm × 10^{-3})	No. of patients	PHA (cpm × 10^{-4})	Pokeweed mitogen (cpm × 10^{-3})	No. of patients	PHA (cpm × 10^{-4})	Pokeweed mitogen (cpm × 10^{-3})
Before RT		57	113 ± 84†	25 ± 22†	40	74 ± 65†	21 ± 22†	24	87 ± 68†	24 ± 28†
Last day of RT		36	67 ± 56‡	8.3 ± 10.8‡	24	40 ± 37	7.4 ± 7.8‡	14	26 ± 33‡	4.3 ± 7.0‡
8 wks after RT	No immunotherapy	15	92 ± 77	22 ± 24	8	40 ± 50	13 ± 14	6	53 ± 58	8.1 ± 12
	Immunotherapy	13	45 ± 35	9.9 ± 10	7	36 ± 46	9.5 ± 10	6	47 ± 31	6.7 ± 6.5
16 wks after RT	No immunotherapy	11	99 ± 88	9.3 ± 8.9	7	33 ± 46	9.9 ± 8.2	6	49 ± 41	8.8 ± 8.4
	Immunotherapy	10	59 ± 39	16 ± 18	4	20 ± 49	3.9 ± 1.3	6	58 ± 30	6.2 ± 4.6
28 wks after RT	No immunotherapy	5	133 ± 124	9.4 ± 6.9	4	37 ± 7	10 ± 9.4	2	93 ± 26	11 ± 15
	Immunotherapy	7	39 ± 35	11.0 ± 11.0	1	12	2.0	4	107 ± 103	25 ± 27

*Control = 47 × 10^3 (93 controls) pokeweed mitogen; control = 160 ± 79 × 10^3 (93 controls) PHA.
†Significantly different from control at $P < 0.05$.
‡Significantly different from before RT at $P < 0.05$.

toward or reached pretreatment levels but not those of control values. The improvement was somewhat greater in those patients who did not receive immunotherapy compared to those treated with BCG and levamisole. A similar improvement toward pretreatment values was not observed in lung cancer patients, possibly indicating that this disease is usually much more generalized than the other two diseases and reduction of tumor burden with RT was not as complete.

Similar data for pokeweed mitogen-induced blastogenesis are also presented in table 2. As with PHA-induced transformation, values at diagnosis are significantly below normal in all three patient groups, with a further significant decrease (by paired analysis) following RT. Only moderate trends toward a return, even to pretreatment values, are seen in any of the groups.

Statistical evaluation by repeated measures analysis of variance to compare immunotherapy versus no-immunotherapy with respect to lymphocyte blastogenesis was again appropriate only for preliminary data. Group means of head and neck carcinoma patients evaluated at all intervals through 16 weeks are presented in figure 3. Trends in these smaller subgroups are similar to those in the larger population. Pre-RT values are somewhat below normal, then decrease dramatically and significantly at the end of RT. Again, there is a suggestion from these preliminary results that recovery is better in the no-immunotherapy group, but differences are not statistically different with the relatively small group that can be evaluated at this time. PHA and pokeweed transformation data for patients evaluated at all intervals through 8 weeks in the lung group are similarly presented in figure 4. In lung patients, initial pretreatment values are strikingly lower than in healthy controls. Because of this the subsequent decrease following RT appears less dramatic, but is nevertheless statistically significant. There is some indication that values are returning at least to pretreatment levels by 8 weeks after RT, especially in the no-immunotherapy group. More data are needed at these and subsequent evaluation intervals before trends can be established.

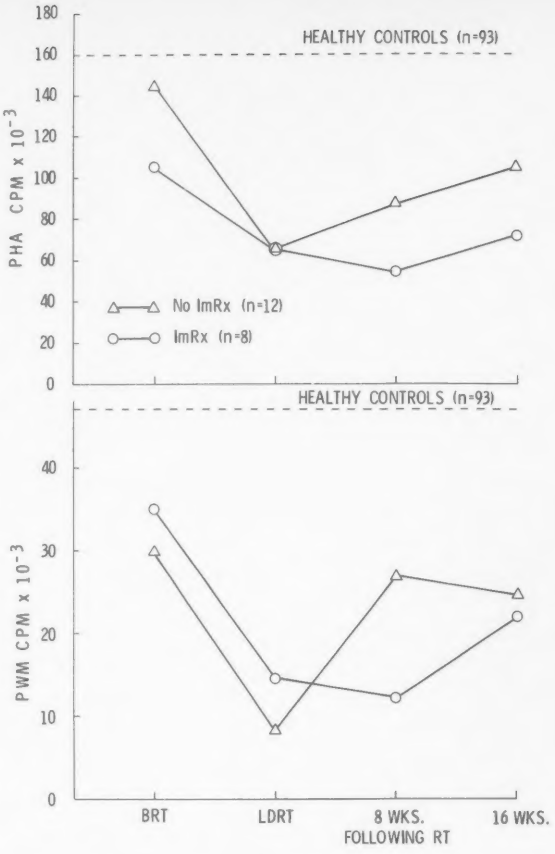

FIGURE 3.—Mitogenic stimulation in head and neck carcinoma patients.

Sialic Acid

Table 3 shows plasma sialic acid levels of the three populations of patients before RT, at the termination of RT, and at subsequent intervals after RT with or without immunotherapy in comparison to normal controls. The mean control value in 52 patients was 0.485 mg/ml with a standard deviation of 0.089. Sialic acid levels in all three groups of patients were significantly increased above the control value to means of 0.623, 0.658, and 0.881 mg/ml respectively for patients with head and neck, cervical, and lung cancer. Patients with cervical cancer showed a further, statistically significant increase to 0.866 mg/ml after RT above the already elevated pre-RT values. Otherwise, these values showed some fluctuations but there was no trend noted other than the fact that those patients receiving immunotherapy tended to have somewhat higher values than their counterparts receiving no immunotherapy.

Lymphocyte Cytotoxicity

Cytotoxic activity of lymphocytes isolated from cancer patients was investigated by incubating them for 48 hours with two human tumor target cell lines: FaDu, a squamous cell carcinoma of the

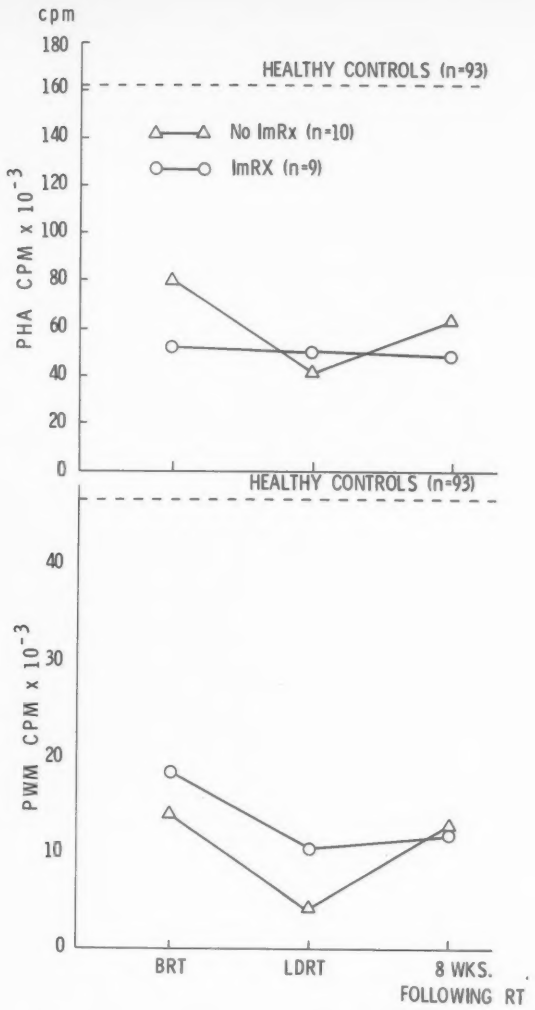

FIGURE 4.—Mitogenic stimulation in lung carcinoma patients.

pharynx, and SK-MES-1, a squamous cell carcinoma of the lung. Results were expressed as percent cytotoxicity and were evaluated by determining whether cytotoxic activity in a given patient had increased, decreased, or remained unchanged when compared with such activity at an earlier interval in the study. Change was defined as a difference of $> 10\%$ cytotoxicity. As can be seen in figures 5 and 6, RT had no clear-cut effect on lymphocyte cytotoxicity in either the head and neck group or the lung group, ie, similar numbers of patients showed increases and decreases and 15%–30% of the patients showed no change. Lymphocyte cytotoxic activity at 8 weeks after RT, however, tended to be increased in a significant proportion of the patient population when compared to cytotoxicity at either the end of or prior to RT. Because of the relatively small number of patients evaluated at this time, a statistical comparison of immunotherapy versus no immunotherapy was not possible. There is some indication, however, that immunotherapy stimulates cytotoxic activity in that 85% of the immunotherapy group (both head and neck and lung groups) showed increased cytotoxicity at 8 weeks when compared to pre-RT levels, whereas only 66% of the patients receiving no immunotherapy showed an increase. The significance of increased, decreased, or unchanged cytotoxic activity with respect to clinical progress will be evaluated as the study progresses.

Skin Tests

For comparison, table 4 summarizes the numbers of patients in various populations tested before RT, at the completion of RT, and at various intervals following RT, and indicates percentage who reacted positively to each skin test. The percentages of anergic patients remained approximately the same before RT and at the completion of RT in all populations of patients for all recall antigens. Patients were sensitized with 2000 μg of DNCB prior to RT and therefore the observed increases in positive responses to DNCB challenge were expected. For most skin tests there was a significant increase in the percentages of positive reactions at 8 weeks after RT. Unlike other parameters of immunocompetence, the results did not appear to differ much between those who had received and those who had not received immunotherapy, except for the PPD test which would be expected to show a marked difference between those receiving BCG and those receiving no immunotherapy. The numbers are too small for comment at 16 weeks after RT.

DISCUSSION

The purpose of a present study was to evaluate the effects of levamisole and BCG administration on various aspects of cellular and humoral immunity in patients undergoing radical RT for head and neck, lung, or cervical cancer. The results indicate that RT further decreases the already impaired immunocompetence of the patients. Preliminary data suggest that administration of BCG and levamisole tends to delay the increase of various immunologic parameters in the immunotherapy group compared with the no-immunotherapy group.

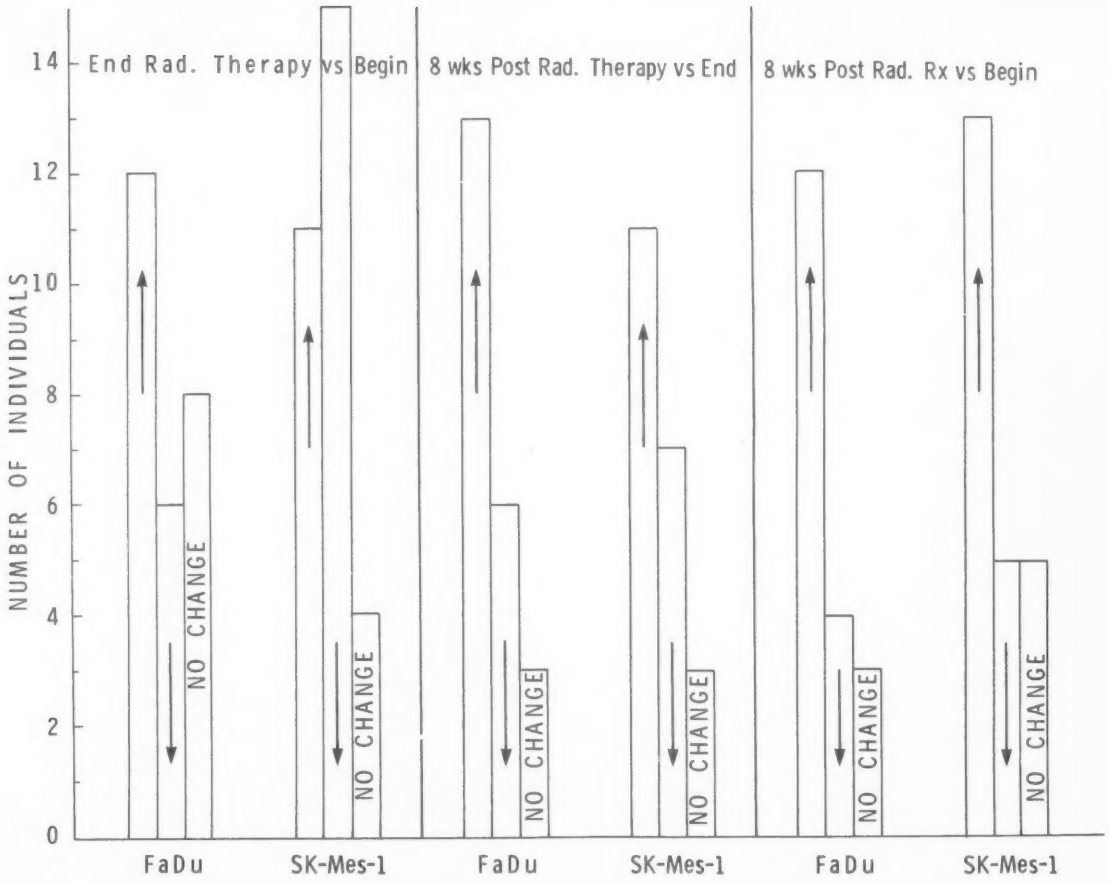

FIGURE 5.—Changes in lymphocyte cytotoxicity values in head and neck carcinoma patients.

Our previous studies (8) demonstrated that 6–8 weeks of adjuvant therapy with levamisole in patients with melanoma and squamous cell carcinoma after curative surgery resulted in improved immunocompetence in clinical responders. Patients who developed metastases or recurrences tended to have continued impairment of immunity. Use of BCG and levamisole for 8 weeks following curative RT does not seem to improve patients' immunocompetence in comparison to no-immunotherapy patients in the present study. It is possible that patients who had radical RT recover more slowly, but longer observation periods are required before any meaningful conclusions can be drawn. The degree of impairment of immunity also tends to be greater following RT than following surgery and may account for the differences between the two studies. Combined immunotherapy with BCG and levamisole may also produce different results than adjuvant therapy with levamisole alone.

Although observation times in the present study are not yet sufficient for an evaluation of the effects of levamisole and BCG adjuvant therapy on metastasis, recurrence, or survival rates in these RT patients, several previous studies have indicated that such therapy may be beneficial under certain conditions. BCG has been extensively evaluated as a nonspecific potentiator of cellular immunity acting through T-cell and macrophage stimulation (9).

A study combining RT with adjuvant immunotherapy was reported by Pines (10) in 1976 in which 48 patients with regional squamous cell carcinoma

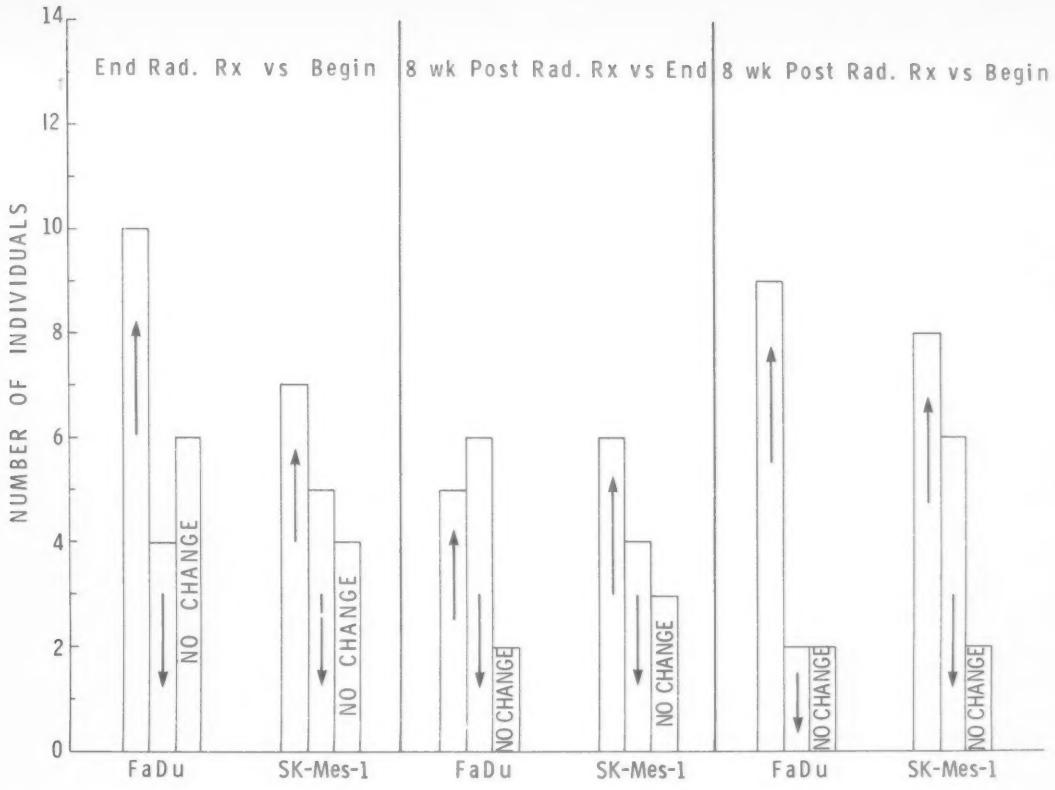

FIGURE 6.—Changes in lymphocyte cytotoxicity values in lung carcinoma patients.

TABLE 3.—Plasma sialic acid levels*

Time	Immunotherapy	Carcinoma					
		Head and neck		Lung		Cervix	
		No. of patients	Mean level (mg/ml)	No. of patients	Mean level (mg/ml)	No. of patients	Mean level (mg/ml)
Before RT		52	0.623 ± 0.123†	40	0.881 ± 0.212†	23	0.658 ± 0.234†
Last day of RT		36	0.613 ± 0.138	24	0.742 ± 0.172	12	0.866 ± 0.281‡
8 wks after RT	No immunotherapy	15	0.675 ± 0.179	8	0.796 ± 0.297	6	0.637 ± 0.150
	Immunotherapy	13	0.653 ± 0.169	6	0.912 ± 0.234	6	0.668 ± 0.214
16 wks after RT	No immunotherapy	11	0.565 ± 0.079	7	0.780 ± 0.271	5	0.646 ± 0.130
	Immunotherapy	10	0.623 ± 0.162	4	0.759 ± 0.132	6	0.693 ± 0.219
28 wks after RT	No immunotherapy	5	0.588 ± 0.108	4	0.759 ± 0.131	2	0.513 ± 0.066
	Immunotherapy	8	0.717 ± 0.232	1	0.727	4	0.769 ± 0.146
40 wks after RT	No immunotherapy	3	0.649 ± 0.227				
	Immunotherapy	3	0.563 ± 0.061				

*Controls = 0.485 ± 0.089 (52 controls).
†Significantly different from control at $P < 0.05$.
‡Significantly different from before RT at $P < 0.05$.

TABLE 4.—Skin test results*

Antigen	Before RT		After RT		8 wks after RT				16 wks after RT			
					No immunotherapy		Immunotherapy		No immunotherapy		Immunotherapy	
	No. of patients tested	% positive	No. of patients tested	% positive	No. of patients tested	% positive	No. of patients tested	% positive	No. of patients tested	% positive	No. of patients tested	% positive
Head and neck												
Candida	47	30	29	34	9	67	5	80	3	67	6	50
Mumps	44	36	28	32	9	44	5	0	2	50	6	33
PPD	49	28	29	21	9	22	5	80	3	33	6	67
Streptokinase	57	61	38	63	17	94	14	86	11	100	10	80
DNCB (100 μg)	42	17	20	40	5	60	7	57	2	50	2	100
Lung												
Candida	37	24	22	45	7	14	3	100	6	33	2	0
Mumps	37	40	22	32	7	43	4	0	6	33	2	50
PPD	36	28	22	32	7	14	3	67	6	17	2	100
Streptokinase	40	30	26	58	8	50	7	71	7	28	4	75
DNCB (100 μg)	37	5	17	29	5	40	4	25	3	0	1	0
Cervix												
Candida	22	27	14	28	6	50	5	60	6	50	6	17
Mumps	23	30	14	28	6	33	5	20	6	33	6	17
PPD	22	14	14	21	6	17	5	100	6	17	6	67
Streptokinase	24	50	16	44	6	67	6	67	6	67	6	33
DNCB (100 μg)	20	0	6	17	5	80	5	40	4	50	5	40

*Actual dimensions for both erythema and induration are recorded but were not analyzed at this time. Criterion for positive skin test was erythema or induration > 10 × 10 mm. Data represent group analysis at each interval. Changes within individual patients will be analyzed in detail when the study has progressed further.

of the lung received RT (4000–6000 rads in 4–5 weeks) before being randomized to BCG (Glaxo strain) at a dose of 25–125 $\times 10^6$ organisms by scarification (Heaf gun) weekly or biweekly or to no further treatment. At 12 months from the start of immunotherapy, 20 of 25 BCG-treated patients were alive, compared to 11 of 23 patients receiving no immunotherapy ($P < 0.005$). Three of 25 patients were still alive at 5 years in the BCG group while there were no survivors in the group receiving no immunotherapy. The recurrence pattern was also different: 12 of 22 BCG-treated patients and six of 23 controls had local recurrence, while two of 22 BCG-treated patients and 12 of 23 controls had extrathoracic relapse, with or without local recurrence.

McKneally and Maver (11) used intrapleural BCG vaccination following curative surgery for lung carcinoma. Patients randomized for immunotherapy received a single dose of 10^7 units of Tice strain BCG intrapleurally in the immediate postoperative period. Fourteen days following injection of BCG, isoniazid (300 mg/day) was begun and was continued for 12 weeks. They found a significant reduction in the incidence of tumor recurrence in stage I patients treated with intrapleural BCG ($P = 0.009$). Two of 30 stage I patients developed recurrences, one developed metastases, and one developed a new lesion. In comparison, of 33 stage I control patients treated with isoniazid alone, 11 have developed recurrent cancer. In stage II and III patients, 13 of 22 BCG-treated patients had recurrence compared to eight of 15 control patients. The authors concluded that patients with more extensive cancer in stage II and III derived no detectable benefit from BCG in contrast to the beneficial effects of adjuvant immunotherapy in stage I disease.

In both studies above (10,11), improved immunocompetence as measured by skin tests was found in clinical responders. The results of these studies are especially impressive in stage I disease. Since our earlier studies suggested that levamisole potentiated the immune response of lymphocytes, probably through the cyclic nucleotide systems, and improved the general immunocompetence of patients with melanoma and squamous cell carcinoma of the head and neck (8,12), we decided to use both BCG and levamisole as adjuvant therapy.

Clinical observations on lung cancer patients who received both levamisole and BCG were recently published (13). Intrapleural BCG (Tice strain, 10^7 viable colony-forming units administered on the 4th day after surgery) and levamisole (2.5 mg/kg orally given 3 days prior to surgery and continued for 2 consecutive days of each week for 18 months or until relapse) were administered to 52 patients with non-oat cell carcinoma of the lung considered fully resectable and potentially cured by surgery alone. Only two of 11 patients in an initial trial and four of 52 in a prospective, randomized trial have shown tumor recurrence with 289 days of observation time. A higher conversion rate of PPD skin tests was observed in patients receiving levamisole and intrapleural BCG compared to patients receiving intrapleural BCG alone, suggesting that levamisole may augment the host response to BCG. None of their 16 patients with PPD conversion has shown tumor recurrence after 289 days of observation.

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Randomized Trial of Levamisole in Patients With Squamous Cancer of the Head and Neck: A Preliminary Report [1]

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SUMMARY

This is a preliminary report of a randomized double-blind study comparing levamisole with placebo as surgical adjuvant treatment of patients with squamous cancer of the head and neck. The study groups were comparable according to the prognostic factors of type (primary or recurrent), site (oral cavity, pharynx, or larynx), and stage of disease and were also similar in pretreatment immune function and in radiation exposure. Of 65 evaluable patients, 31 received levamisole and 34 received placebo. Although there was no difference in the distribution of the time to recurrence of the overall treatment group, there was a difference of borderline significance in favor of levamisole in the primary disease patients ($P < 0.06$). Further analysis of subgroups in the primary disease category showed decreased recurrence rates in patients receiving levamisole who had cancer of the oral cavity ($P < 0.01$) or who had stage II disease ($P < 0.02$). Considering site and stage simultaneously, the only significant difference was in stage II patients with oral cancer. The above results, although encouraging, must be viewed with caution due to small sample sizes and the relatively short followup of most patients.

[Cancer Treat Rep 62:1663–1669, 1978]

In patients with squamous cell carcinoma of the head and neck, recurrence at the primary site or in the neck is the most common cause of death. The rate of recurrence depends on site of primary, stage of disease, and whether or not there has been prior surgery. In the study by Farr and Arthur of 1405 patients with squamous cancer of the mouth and pharynx at Memorial Sloan-Kettering Cancer Center, the recurrence rate ranged from 39% for patients with cancer of the anterior tongue to 73% for those with disease of the base of the tongue (1). The 5-year survival rate ranged from 42% for primary cases to 20% for those with prior surgery; the 5-year survival was 89% for patients with stage I disease, 59% for stage II, 33% for stage III, and 8% for stage IV.

A second major feature of this disease is early and marked immunosuppression (2–6). Approximately 30%–50% of patients have depressed immune function as demonstrated by skin tests and in vitro lymphocyte function studies prior to treatment (3,6). Furthermore, a significant correlation has been observed between the recurrence rate and the dinitrochlorobenzene (DNCB) response in patients with stage I and II cancer (3).

Levamisole is a phenylimidazothiazole anthelmintic agent which was found to augment the response to *Brucella* vaccination in mice (7,8). Since then, a rapidly expanding literature has documented the immunopotentiating effects of this compound (reviewed by Symoens in ref 9).

A controlled clinical trial in patients with lung

[1] Supported by contract N01-CB-53875 (Division of Cancer Biology and Diagnosis) from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, and generous funds from the Cancer Research Institute (New York). Dr. Wanebo was a recipient of the American Cancer Society Junior Clinical Faculty Fellowship.

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[4] We are grateful to Dr. John Garofalo, Cynthia Kosloff, Mona Sussman, and JoAnne Sweeney for their assistance in carrying out this study.

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cancer has suggested that there may be antitumor effects of levamisole in man (10,11). In addition, Rojas et al reported significant beneficial effects of levamisole on recurrence rates and survival in a double-blind study comparing levamisole with placebo as adjuvants to radiotherapy for stage III and IV breast cancer (12,13). We began a prospective, randomized, double-blind trial of levamisole in patients with squamous cell carcinoma of the head and neck region in July 1975. A preliminary analysis of that study is reported here.

MATERIALS AND METHODS

Patient Accession and Randomization

Between July 1975 and July 1978, 80 patients were entered into the study, of whom 65 were evaluable. All patients had squamous cancer of the oral cavity, pharynx, or larynx, and had been stratified according to type of disease (primary or recurrent), site of primary, and stage of disease, prior to being randomized to receive levamisole or placebo. There were 31 evaluable patients in the levamisole group and 34 in the placebo group. The distribution of patients in the various strata is shown in table 1.

TABLE 1.—Clinical characteristics

Characteristic	Treatment group	
	Levamisole (31 patients)	Placebo (34 patients)
Type of disease		
Primary	24	29
Recurrent	7	5
Site of primary		
Oral cavity	23	22
Pharynx	1	5
Larynx	6	6
Unknown	1	1
Stage of disease		
I	7	7
II	6	8
III	17	18
IV	1	1

Treatment

After randomization, treatment with 150 mg of levamisole or placebo three times weekly on alternate weeks was carried out for 2 years, unless recurrence or excessive toxicity occurred.

Immunologic Methods

Most patients were skin-tested and had lymphocyte counts and lymphocyte function studies performed prior to initiation of the study. Patients were tested for pre-existing delayed hypersensitivity to Dermatophyton-0 (1:100, Hollister Stier Laboratories, Yeadon, Pa), mumps skin-test antigen (Eli Lilly and Co, Indianapolis, Ind), intermediate strength tuberculin (Parke-Davis Co, Detroit, Mich), and streptokinase/streptodornase (Lederle Laboratories, Pearl River, NY). The antigens were injected intradermally in volumes of 0.1 ml. The tests were read at 48 hours and considered positive when the diameter of induration was $\geq$ 5 mm.

Sensitization and challenge with 2,4-DNCB was carried out according to a modification of the method of Eilber and Morton (4,14). DNCB at a dose of 2000 μg dissolved in 0.1 ml of acetone was applied to the inner aspect of the upper arm, within the confines of a plastic ring measuring 2 cm in diameter, and allowed to evaporate. Prior hypersensitivity to DNCB was excluded by control testing with 100 and 25 μg of DNCB in 0.1 ml of acetone applied simultaneously to the ipsilateral forearm. All test sites were covered for 48 hours, kept dry, and examined for erythema and induration at 2 days and 2 weeks. If there was no reaction at the 100- and 25-μg sites, the patients were rechallenged at 2 weeks on the ipsilateral extremity with 100, 50, and 25 μg of DNCB. The reaction was considered positive if there was induration of $\geq$ 5 mm 48 hours later.

Lymphocyte transformation in vitro was performed using a micro-method (6). Lymphocytes were obtained from heparinized blood on Ficoll-Hypaque ("Lymphoprep") density gradients, and resuspended in RPMI-1640 with Hepes buffer, glutamine (0.25 ng/ml), penicillin (10 units/ml), streptomycin (10 units/ml), heparin (10 IU/ml), and 15% pooled normal human serum. Lymphocyte culture was performed in flat-bottom microtiter plates (Falcon #3040) by adding 100,000 lymphocytes in 200 λ of medium. Transformation of lymphocytes was measured in terms of incorporation of ^{14}C-thymidine which was added after 72 hours and then incubated at 24 hours. Cultures were performed in triplicate. Dilutions of mitogens which had been prepared in advance and stored at −20°C were freshly thawed and added to the cultures in 25-λ volumes. Concentrations of phytohemagglutinin at 500, 250, 50, 10, and 5 μg/ml, concanavalin A (Difco Labs, Detroit, Mich) at 125, 42, 15, and 5 μg/ml, and pokeweed mitogen (Gibco, Grand Island, NY) at 2000, 400, and 80 μg/ml were used.

Statistical Methods

Curves for time to recurrence were calculated by the Kaplan-Meier product limit procedure (15). For the comparison of the distributions of times to recurrence for two or more groups, the log rank test was applied (16). This test can also be used to detect differences in distributions for the groups while controlling for other factors thought to be associated with prognosis. All reported P values refer to two-sided tests.

TABLE 2.—Pretreatment immunologic characteristics

Characteristic	Treatment group	
	Levamisole	Placebo
DNCB	6/27*	8/30
Absolute lymphocyte count	8/21	8/20
Lymphocyte response to—		
Phytohemagglutinin	15/22	11/21
Concanavalin A	16/22	11/21
Pokeweed mitogen	12/21	13/18

* No. of patients with abnormal values/No. tested.

RESULTS

Pretreatment Immunologic Characteristics

Most patients had immunologic tests performed prior to enrollment in the study (table 2). Fourteen of 57 patients tested (25%) were DNCB-negative. The lymphocyte count was $< 1200/mm^3$ in 16 of 41 patients (39%). The response of isolated lymphocytes to phytohemagglutinin was < 15,000 cpm in 26 of 43 patients (60%), the response to concanavalin A was < 10,000 cpm in 27 of 43 patients (63%), and the response to pokeweed mitogen was < 5000 cpm in 25 of 39 patients (64%). These cut-off points were chosen somewhat arbitrarily at the 10th percentile of expected responses in lymphocyte cultures from normal controls. There was no significant difference between the levamisole and placebo group with respect to these pretreatment immunologic characteristics. Not enough patients have been studied serially for meaningful analysis of change during therapy.

Recurrence

Statistical analysis was carried out on the recurrence experience of 65 evaluable patients, all of whom had at least 1 month of followup; 53 of these patients had had no definitive surgery prior to entry into the study. The levamisole and placebo groups were compared as a whole, but a more extensive analysis focused on the 53 primary operable patients. The data were considered in terms of disease stage, disease site, and pretreatment DNCB status.

The curves for time to recurrence are shown in figure 1 for the two groups of primary patients. The observed difference in distribution of recurrence times has borderline statistical significance ($P < 0.06$); the corresponding statistical analysis for the entire group of 65 patients yielded a value of $P > 0.1$.

Comparison of the levamisole and placebo groups by site revealed a statistically significant difference in favor of levamisole for patients with cancer of the oral cavity ($P < 0.01$); the corresponding curves are shown in figure 2. There was no significant difference with respect to cancer of the pharynx or larynx; the overall difference, after adjustment for site of disease, was similarly not significant.

For the primary operable patients, there were no recurrences in stage I and IV disease (ten and two patients, respectively). The curves for stage II and III disease are shown in figure 3. In stage II patients there was a statistically significant difference, again in favor of levamisole ($P < 0.02$). The observed difference for stage III patients was not significant, and the overall significance level, after adjustment for stage, was borderline.

It is important to note that among stage II patients, four of five in the levamisole group and five of six in the placebo group had cancer which originated from the oral cavity. When the data were further broken down according to site *and* stage, only one significant difference emerged, and this was for the "oral site, stage II" subgroup.

Figure 4 shows the curves for time to recurrence for patients grouped according to pretreatment DNCB status. These groups are not significantly different. Controlling for other covariates and comparison of the levamisole and placebo groups with adjustment for DNCB again yielded no significant differences.

Toxicity From Levamisole

There was considerable minor toxicity associated with this protocol. Of 65 evaluable patients, 40 (62%) experienced some subjective toxicity and most patients had more than one side effect (table 3). The major side effects were centered in the gastrointestinal and central nervous systems. Treatment was stopped in five patients (four from the levamisole and one from the placebo group). Diarrhea in three of these patients and hives in one were the primary

TABLE 3.—Toxic effects

Toxic effect	No. of patients	
	Levamisole	Placebo
Fatigue	12	3
Bitter taste	7	6
Headache	6	6
Dizziness	8	4
Diarrhea	7	3
Itching	6	5
Insomnia	7	3
Nausea	5	5
Vomiting	3	3
Lightheadedness	6	3
Abdominal cramps	4	3
Belching	5	3
Weakness	6	1
Rash	5	2
Queasiness	4	1
Other	7	1

reasons for discontinuance of therapy. Marked granulocytopenia, which reversed after stopping levamisole, was seen in one patient. It is remarkable how frequent side effects were in the placebo treated patients; 17 of 34 had at least one side effect, compared with 23 of 31 in the levamisole group.

Radiation

Although patients were not specifically stratified according to radiotherapy, approximately the same proportion had received either preoperative or postoperative radiation. There were 12 patients who had received radiation in the placebo group (ten preoperative and two postoperative). In the levamisole group there were 14 patients who had been treated with radiation (ten preoperative and four postoperative).

DISCUSSION

This is a preliminary report of a randomized double-blind study comparing levamisole with pla-

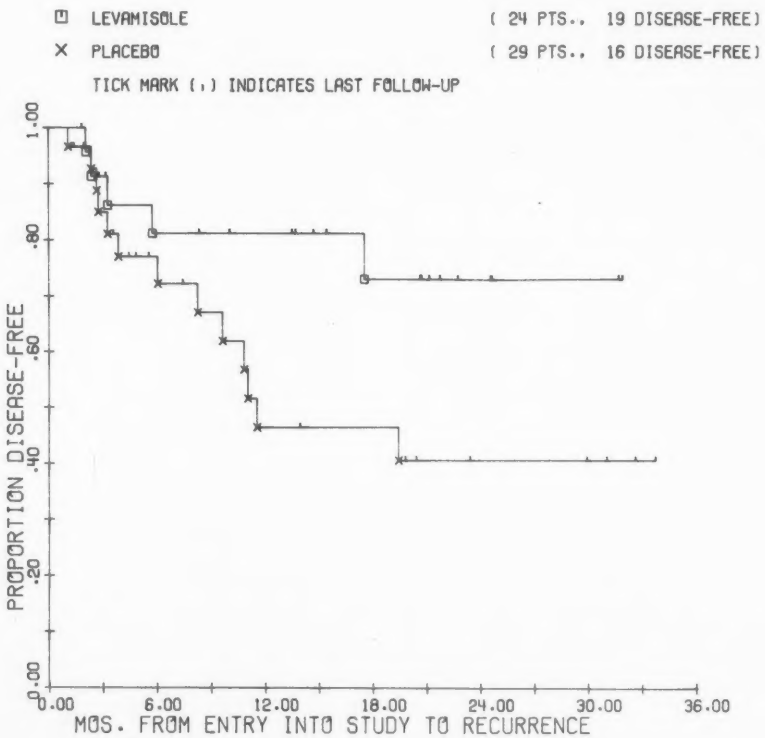

FIGURE 1.—Recurrence distributions of patients with no prior definitive surgery. The advantage for the levamisole-treated patients has borderline statistical significance ($P < 0.06$).

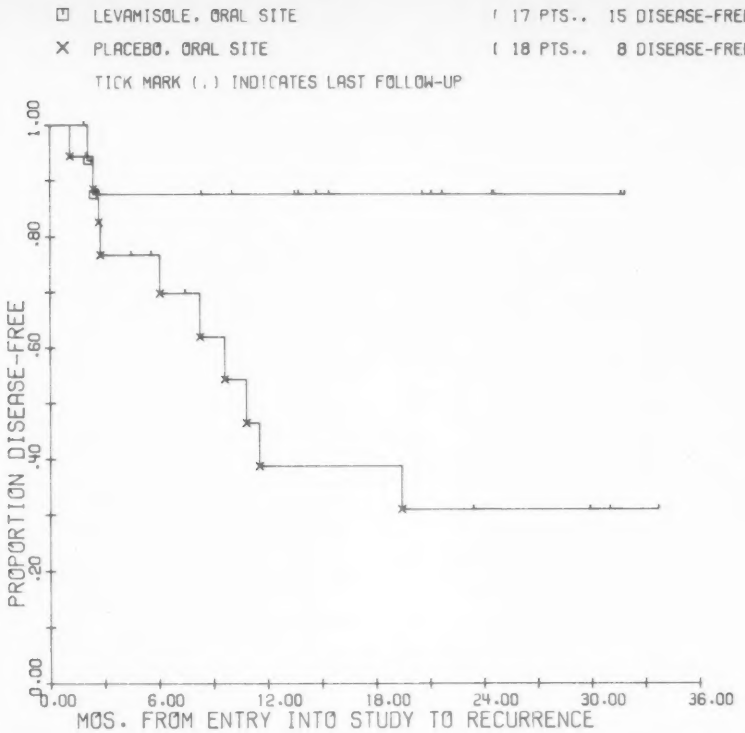

FIGURE 2.—Recurrence distributions of patients with oral cavity cancer who had no prior definitive surgery. The time to recurrence is significantly longer in the patients who received levamisole ($P < 0.01$).

cebo as surgical adjuvant treatment of head and neck cancer patients. The study groups were comparable with respect to selected prognostic factors: type, site, and stage of disease and pretreatment immune function (DNCB, lymphocyte count, and mitogen response).

The difference in the distribution of times to recurrence of the entire group of levamisole and placebo patients was not significant; the comparison for primary disease patients yielded borderline significance. Analysis of the data for the primary patients by site and stage revealed some suggestive differences within these subgroups. In particular, there was a significant difference in recurrence rates in favor of levamisole for patients with cancer of the oral cavity and for stage II patients. Considering site and stage simultaneously resulted in one significant difference, for stage II patients with oral cancer. No differences were found related to pretreatment DNCB status.

Due to the relatively small sample sizes involved in the various subgroups and the fact that many significance tests were carried out in the course of this analysis, the results reported must be interpreted with caution, in the context of exploratory data analysis.

It is remarkable that most of the patients experienced minor side effects. In particular, the development of side effects in half of the placebo treated patients should be noted. On the other hand, only five patients had toxic effects which were severe enough to discontinue treatment (and one of these patients was receiving placebo).

It has been reported that patients with operable squamous cell carcinoma are frequently immunodeficient (2–6). The results of this study once again confirm that observation. The lack of correlation between DNCB skin test results and recurrence is at variance with our previous experience (3,4). This discrepancy is not as surprising as it seems since the correlation was only seen in stage I and II patients in our previous studies (3), and few recurrences have occurred in such patients to date in the present study.

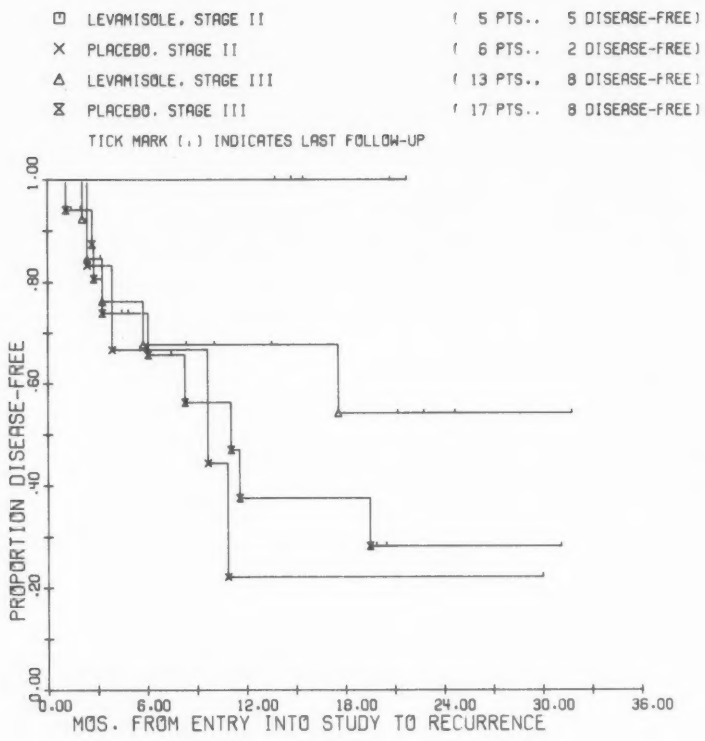

FIGURE 3.—Recurrence distributions of patients with stage II or III disease. For stage II patients, the time to recurrence is significantly longer in those who received levamisole ($P < 0.02$); the difference in patients with stage III disease is not statistically significant.

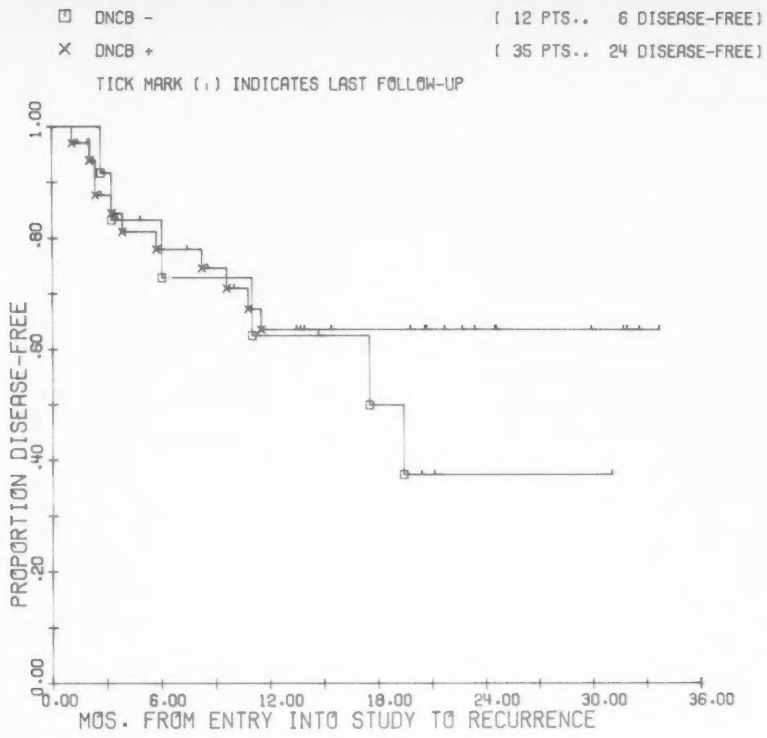

FIGURE 4.—Recurrence distributions according to pretreatment DNCB skin-test reactivity. No difference is seen.

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Preliminary Results of Combined Surgery and Adjuvant Bacillus Calmette-Guérin Plus Levamisole Treatment of Resectable Lung Cancer [1]

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SUMMARY

The administration of intrapleural bacillus Calmette-Guérin (BCG) alone and intrapleural BCG plus levamisole was compared to placebo in patients with resectable, non-small cell cancer of the lung. This report is based on an interim analysis of 100 eligible patients with a median followup of 245 days. No significant treatment effects are apparent at the present time, although current trends are consistent with an approximate 30% reduction in recurrence rate in patients receiving intrapleural BCG. There is little evidence to suggest that the addition of levamisole will contribute to this effect. A relationship between purified protein derivative conversion and tumor recurrence is apparent. Failure to manifest purified protein derivative conversion following administration of intrapleural BCG is associated with a significantly greater risk of tumor recurrence.

[Cancer Treat Rep 62:1671–1675, 1978]

This paper represents an interim analysis of a prospective, double-blind randomized clinical trial to evaluate the effects of intrapleural bacillus Calmette-Guérin (BCG) and intrapleural BCG plus levamisole in patients with resectable non-small cell cancer of the lung. The protocol design is outlined in figure 1. An earlier report of this study has been published elsewhere (1). The use of intrapleural BCG is based on evidence reported by McKneally et al indicating that the administration of intrapleural BCG in patients with stage I lung cancer results in an increased disease-free remission duration and improvement in survival (2–4). The use of levamisole derives from the studies of Amery et al suggesting that lung cancer patients receiving levamisole had fewer recurrences and fewer deaths than control patients receiving placebo (5,6).

Earlier studies have suggested that BCG and levamisole may have different mechanisms of action. BCG has been shown to manifest significant local and regional antitumor effects. Tumor growth is most effectively inhibited when BCG is given as an admixture with tumor cells or delivered directly to the tumor site. Tumor regression following the direct intralesional inoculation of BCG has been documented in animal tumor model systems (7,8) and in man (9).

By contrast, levamisole has been reported to possess systemic antitumor activity (10). Renoux and Renoux reported that mice receiving levamisole

[1]Supported by contract N01-CM-77148 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, and sponsored by the Committee on Lung Cancer of the Northwest Oncology Group.

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[3]We gratefully acknowledge the participation of Drs. Phillip Jolly of the Mason Clinic; Howard Kellogg, Waldo Mills, William B. Hutchinson, Robert Coe, Lloyd King, and Howard Anderson of the Swedish Hospital Medical Center; George Thomas and Thomas Jones of Providence Hospital; Hubert Radke, Wesley Sikkema, Eugene Hessel, and Douglas Johnson of the University of Washington-affiliated hospitals who have entered patients in this protocol; Drs. Terry Rogers, Leonard Hudson, Jon Huseby, Ed Morgan, Richard Winterbauer, John Allen, Neely Pardee, and S. Laksminarayan, pulmonary disease consultants; Drs. Harold Hall, Bruce Kulander, Tom Norris, David Thorning, Ed Barker, and Sam Hammer, pathology consultants; and the pharmacy staffs of the Mason Clinic, Swedish Hospital Medical Center, Providence Hospital, and the University-affiliated hospitals in Seattle.

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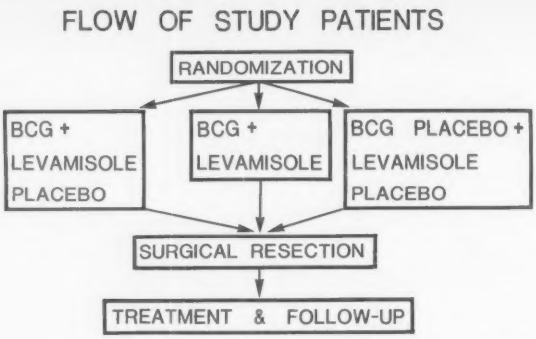

FIGURE 1.—Outline of protocol design.

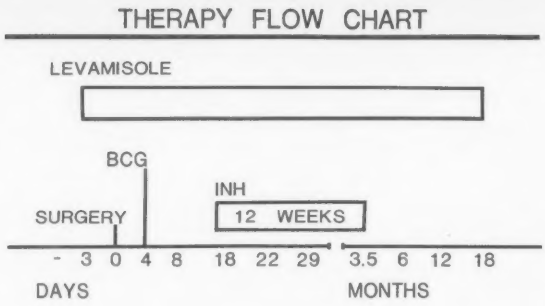

FIGURE 2.—Treatment schema.

and inoculated with the transplantable syngeneic Lewis lung tumor had a significant reduction in pulmonary metastases when compared to controls (11). Little difference in the growth of tumor at the implantation site was noted, however, between the levamisole and control groups. Similar observations have been reported in other animal model systems (12) and in patients with lung cancer. Amery found that patients receiving levamisole had a reduced incidence of distant metastases whereas the incidence of intrathoracic recurrence was virtually identical in patients receiving levamisole or placebo (5,6). Since BCG and levamisole may act by independent mechanisms (BCG to limit local tumor growth and levamisole to limit distant metastases), these two agents have been administered together in the present study to evaluate the possibility that their combined effects may be additive or synergistic.

MATERIALS AND METHODS

Patients

Patient eligibility and staging have been previously described (1). In brief, patients with histologically proven epidermoid carcinoma, adenocarcinoma, or undifferentiated large cell carcinoma of the lung, considered fully resectable and potentially curable by surgery alone, were eligible for study. Histologic evaluation of the surgical specimen must have shown no evidence of mediastinal lymph node involvement or tumor at cut margins. Patients must have received no preoperative chemotherapy, immunotherapy, or radiation therapy. Patients with serious concomitant disease, inadequate pulmonary function, or a history of active tuberculosis within 5 years were excluded.

Prior to surgery, a cbc, chemical screening battery, liver function studies, pulmonary function studies, and chest roentgenogram were obtained from all patients. Isotope scanning was done when indicated by symptoms or laboratory abnormalities. Mediastinoscopy or mediastinotomy, although not required, was generally performed on patients with large or centrally located primary tumors. All patients were staged prior to surgery according to a clinical TNM classification (13,14).

Immunologic evaluation included skin tests with a defined battery of "recall" antigens and dinitrochlorobenzene sensitization and challenge in all patients.

Randomization and Therapy

Prior to surgery, patients were stratified according to the clinical TNM classification and assigned to one of three possible treatment groups using an unbalanced randomization scheme. All patients except stage III patients had (*a*) a 40% chance of being assigned to immunotherapy with intrapleural BCG and levamisole; (*b*) a 40% chance of being assigned to immunotherapy with intrapleural BCG and levamisole placebo; and (*c*) a 20% chance of being assigned to receive intrapleural BCG placebo and levamisole placebo. Stage III patients were equally allocated between the two nonplacebo treatments. It has been estimated that approximately 220 patients must be randomized in the three treatment groups to detect a 40% reduction in the death rate with a probability of 0.95.

Levamisole at a dose of 2.5 mg/kg (or its placebo) was given for 3 consecutive days prior to surgery and continued for 2 consecutive days of each week for 18 months or until relapse. Intrapleural BCG (Tice strain, 10^7 viable colony-forming units) was administered on approximately the fourth postoperative day, as described by McKneally et al (3).

TABLE 1.—Patient characteristics for 100 eligible patients

Men	65
Women	35
Median age in yrs	65
Range	45–75
No. of patients with—	
Cell type	
Adenocarcinoma	45
Epidermoid carcinoma	29
Large cell undifferentiated	10
Mixed	9
Other	7
Stage and TNM classification (pathologic)	
I $T_1N_0M_0$	30
$T_2N_0M_0$	33
$T_1N_1M_0$	11
II $T_2N_1M_0$	13
III $T_3N_0M_0$	4
$T_2N_1M_0$	3
$T_{1-3}N_2M_0$	6

Two weeks following BCG administration, Isoniazid (INH), at a dose of 300 mg/day, was given for 12 consecutive weeks. Patients receiving intrapleural BCG placebo received INH placebo. Patients were evaluated at 6 and 12 weeks, and then at three monthly intervals following surgery. The treatment schema is shown in figure 2.

CLINICAL RESULTS

This report is based on an evaluation of 100 patients judged eligible after surgery and followed for at least 3 months. Patients were randomized between March 8, 1976, and January 15, 1978, when this analysis was conducted. The median patient followup has been 245 days. Patient characteristics are summarized in table 1.

Objective tumor recurrence has been documented in 22 patients. Recurrence has been noted in 12 of 46 (26%) patients, in five of 28 (18%) patients, and in five of 26 (19%) patients receiving intrapleural BCG plus levamisole, intrapleural BCG alone, and placebo, respectively. A detailed analysis of treatment results has been undertaken using the proportional hazards model formulated by Cox (15). This model presumes that the rate of recurrence can be written as a multiplicative function of prognostic factors, ie, performance status, tumor size, surgical stage, and treatment (intrapleural BCG or levamisole or both) were considered in the present analysis, and the model uses the data to estimate the multiplicative effect of each factor on the rate of recurrence in the presence of all of the others.

TABLE 2.—Maximum likelihood fit of proportional hazards (Cox, ref 15) model to recurrence data

Treatment	Estimated % reduction in recurrence rate
BCG	27 (P = 0.65)
BCG + levamisole	32 (P = 0.52)

Using this approach, it is possible to estimate the effect of treatments with BCG or levamisole on recurrence rates while controlling for the other factors, eg, performance status, tumor size, and surgical stage, that may influence the recurrence rate. Results of this analysis are shown in table 2.

No significant treatment effects are apparent at the present time. If current trends are confirmed by future data, however, these results are consistent with approximately a 27% and 32% reduction in recurrence rates in patients receiving intrapleural BCG alone and intrapleural BCG plus levamisole, respectively. It should be emphasized that the confidence intervals are large. At a level of 95% confidence, the present data are consistent with BCG having an effect ranging from a *reduction* in recurrence rate of 80% to an *increase* in recurrence rate of 180%. Similarly, the data are consistent with BCG plus levamisole having an effect ranging from a *reduction* in recurrence rate of 80% to an *increase* in recurrence rate of 120%. Although the present results suggest a trend in favor of patients receiving either intrapleural BCG alone or intrapleural BCG plus levamisole, as compared to placebo, they provide no evidence that levamisole increases the antitumor effect.

Skin Test Results

Preoperative and postoperative skin test information has been analyzed according to treatment and prognosis. There is no evidence that the results of any of the preoperative skin tests were predictive of recurrence, with the possible exception of the response to intermediate-strength purified protein derivative (PPD). The present data suggest that patients who are initially unresponsive to PPD (< 10 mm induration) are less likely to develop recurrence than patients reactive to PPD (P = 0.13).

In patients who were PPD-negative prior to surgery, it has been possible to evaluate the frequency of conversion to positive PPD reactivity following the postoperative administration of intrapleural BCG (or its placebo). These data are summarized in table 3.

Thirty-seven of the 56 patients (66%) who were PPD skin test-negative prior to surgery have shown

TABLE 3.—Relationship of PPD conversion and treatment

Treatment	PPD conversion*		Totals
	Yes	No	
Placebo	3 (30%)	7	10
BCG	16 (84%)	3	19
BCG + levamisole	18 (67%)	9	27
Totals	37 (66%)	19	56

*Patients pretherapy PPD < 10 mm induration; posttherapy PPD ≥ 10 mm induration.

a conversion of their test response to positive postoperatively. PPD conversion was specific for BCG immunotherapy, ie, 34 of 37 (92%) conversions occurred in patients receiving intrapleural BCG. A reduced incidence of conversion in patients receiving levamisole plus BCG (67%) is suggested when compared to patients receiving BCG alone (84%), but the difference between these two treatment groups is not statistically significant ($P = 0.19$).

Further analysis suggests that PPD conversion may be associated with tumor recurrence. The analysis of the association between PPD conversion and tumor recurrence restricted to patients receiving intrapleural BCG demonstrates that there is a significant difference ($P = 0.04$) between the number of recurrences: four of 34 (12%) and five of 12 (42%) of PPD convertors and nonconvertors, respectively. These data are shown in table 4. The results suggest that failure to manifest PPD conversion may be associated with poor prognosis in patients who receive intrapleural BCG. The difference in recurrence rate in PPD convertors and nonconvertors is not explained by differences in surgical stage, tumor size, or cell type.

Earlier published reports have suggested that treatment with levamisole may enhance skin test reactivity, particularly in patients whose reactivity is initially depressed. To evaluate this possibility, preoperative and postoperative skin test results in patients included in the present study were compared. Reactivity to all skin test antigens was increased postoperatively. Increases were no greater, however, in patients receiving levamisole than in placebo controls. Increases in PPD reactivity in patients initially PPD-negative who received intrapleural BCG were comparable whether patients were assigned levamisole or levamisole placebo.

DISCUSSION

On the basis of its previously reported activity as an immunorestorative agent with possible benefit in the therapy for patients with non-small cell

TABLE 4.—Relationship of PPD conversion and prognosis

Patient group	No. of recurrences/patients	
PPD conversion	4/34 (12%)	($P = 0.04$)
PPD nonconversion	5/12 (42%)	

carcinoma of the lung, it could be predicted that levamisole in the present protocol might increase the host response to intrapleural BCG (and thus perhaps enhance the local and regional antitumor effects of BCG) and might itself manifest substantial systemic antitumor activity.

An increase in the host response to intrapleural BCG in patients receiving levamisole might be reflected by a decrease in the incidence of intrathoracic recurrence, particularly in patients with epidermoid carcinomas whose growth appears to be somewhat more likely to remain localized than other forms of non-small cell carcinoma of the lung. An effect of levamisole would also presumably be manifested by an increase in the frequency of PPD conversion or the intensity of PPD reactivity in patients receiving intrapleural BCG. None of these predictions is suggested by current trends in the present study. The frequency of intrathoracic recurrence was comparable in patients receiving intrapleural BCG with or without levamisole. Similarly, the incidence of PPD conversion in patients receiving intrapleural BCG was not significantly influenced by the administration of levamisole. If anything, present trends suggest that levamisole may reduce the frequency of PPD conversion, although this effect is not yet statistically significant ($P = 0.19$).

An increase in systemic antitumor immunity in patients receiving levamisole might be expected to be manifested by a reduced incidence of distant metastases, particularly in patients with adenocarcinoma or large cell undifferentiated carcinoma which are thought to have a higher likelihood for systemic dissemination than squamous cell carcinoma. Such effects of levamisole were in fact suggested in the studies of Amery et al (5,6). Unfortunately, the results of the present study again fail to provide any evidence to support these possibilities. Present trends do not suggest that the incidence of recurrence will be diminished by the administration of levamisole. It should be emphasized, however, that the number of recurrences observed to date is small, and these results do not exclude the possibility of treatment effects of substantial magnitude.

A substantial amount of skin test information has been analyzed. Our results have failed to support earlier reported observations that levamisole increases skin test reactivity.

One result of interest is the apparent relationship between PPD conversion and tumor recurrence. Failure to manifest PPD conversion following the administration of intrapleural BCG has been shown by the present analysis to be associated with a significantly greater risk of subsequent tumor recurrence. These results suggest that the PPD skin test response may be a useful prognostic factor in patients receiving BCG immunotherapy.

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Final Results of a Multicenter Placebo-Controlled Levamisole Study of Resectable Lung Cancer [1]

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SUMMARY

Two hundred and eleven patients with resectable lung cancer have been treated in a double-blind clinical investigation with either levamisole (50 mg three times a day) or a placebo given in 3-day courses starting 3 days before the operation and repeated every 2 weeks. The study was terminated after all patients had been in the trial for 2 years. The dose of levamisole used in this study appears to be sufficient only for patients weighing ≤ 70 kg and, therefore, future studies should make use of an individually adapted dose. Such a treatment is expected to prolong both the disease-free interval and the survival time without causing major side effects in a significant number of patients. This result is achieved primarily by an inhibition of hematogenous dissemination and occurs especially in those patients who had more advanced tumors at the time of the resection.

[Cancer Treat Rep 62:1677–1683, 1978]

This paper summarizes the final analysis of a double-blind study with levamisole in patients with resectable lung cancer. The study was started during the first half of 1972 and was completed by the end of 1977. Since the first interim analysis published in 1975 (1), four more interim evaluations have been performed; the results of these latter evaluations have been reported at scientific meetings (2–5).

MATERIAL AND METHODS

Two hundred and eleven patients were selected for this study. The patients' characteristics are summarized in table 1. The two treatment groups are comparable except for the number of patients with adenocarcinoma; these patients were somewhat more frequent in the control group ($P = 0.06$, chi-square test, two-tailed). In order to assess the preoperative tumor load, the largest tumor diameter was measured after its removal by the surgeon and its fixation by the local pathologist. Also, a category grouping system was adopted to describe the regional extent of the tumor; within this system, category 1a is the only category where there is no evidence that the tumor could have left or actually has left the primary site. Further details about these assessments are given elsewhere (1,3).

Apart from the operation, all patients were treated with double-blind tablets containing either 50 mg of levamisole or a placebo. Treatment assignment was by randomization which was stratified among the three cooperating centers and the medication was strictly double-blind and individually coded. The dosage was one tablet three times a day for 3 consecutive days every second week and was started 3 days before the operation. This treatment was continued for 2 years unless relapse was evident; in that case, appropriate anticancer treatment was given, but until recurrence was proved, any use of cytostatics, corticosteroids, or irradiation treatment was prohibited.

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[1]This report was prepared by Dr. W. K. Amery (*Chairman*) on behalf of the Study Group for Bronchogenic Carcinoma which includes the following investigators: Prof. J. Cosemans, Dr. H. C. Gooszen, Prof. E. Lopes Cardozo, Dr. A. Louwagie, Dr. J. Stam, Prof. J. Swierenga (*deceased*), Dr. R. G. Vanderschueren, and Dr. R. W. Veldhuizen; consultants include: Eng. G. De Ceuster, Dr. L. Desplenter, J. Dony, Prof. A. Drochmans, Dr. P. A. J. Janssen, and Dr. W. Tanghe; the coordinator is E. Denissen.

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TABLE 1.—Patient characteristics

Characteristic	Levamisole group (total No. = 96)	Placebo group (total No. = 115)	Total population (total No. = 211)
Sex (%)			
Men	96.9	95.7	96.2
Women	3.1	4.3	3.8
Median age in yrs (range)	63 (42–77)	62 (40–76)	63 (40–77)
Median weight in kg (range)	72 (53–137)	71 (40–93)	72 (40–137)
% of patients at treatment center in—			
Amsterdam	16.7	19.1	18.0
Leuven	18.7	20.0	19.4
Utrecht	64.6	60.9	62.6
Median erythrocyte sedimentation rate in mm (range)	21 (2–102)	24 (2–115)	22 (2–115)
Initial skin tests*			
Median diameter in mm with PPD (range)	10 (0–30)	10 (0–40)	10 (0–40)
% of patients receiving DNCB (50 μg) with—			
No induration	50.7	60.0	55.7
Induration	49.3	40.0	44.3
% of patients receiving DNCB (100 μg) with—			
No induration	78.8	86.5	82.9
Induration	21.2	13.5	17.1
Median No. of cigarettes/day (range)	20 (0–70)	20 (0–50)	20 (0–70)
% of patients with—			
Tumor location			
Right	55.2	46.1	50.2
Left	44.8	53.9	49.8
Peripheral	53.1	60.0	56.9
Central	46.9	40.0	43.1
Type of surgery			
Pneumonectomy	27.1	29.6	28.4
Lobectomy	70.8	66.9	68.7
Other	2.1	3.5	2.9
Histology			
Squamous cell carcinoma	67.7	60.0	63.5
Adenocarcinoma	14.6	26.1	20.9
Others or mixed type	17.7	13.9	15.6
Regional extent of disease			
Category 1a	54.2	52.2	53.1
Other categories	45.8	47.8	46.9
Largest median tumor diameter in cm (range)	4 (1–15)	4 (1–13)	4 (1–15)
Duration in mins of anesthesia (range)	150 (60–270)	150 (75–270)	50 (60–270)

* PPD = purified protein derivative; DNCB = dinitrochlorobenzene.

RESULTS

The overall death rate was slightly higher in the placebo group than in the levamisole group, ie, 55 and 44 patients, respectively. This is primarily due to a higher incidence of cancer deaths in the control group: 29 patients in the levamisole group versus 43 patients in the placebo group. Since in all previous interim analyses (2–5) several patients were found to have been underdosed in this study, the crude recurrence and death rates were re-analyzed with respect to the relative dose (expressed as mg/kg) the patients had taken. This analysis is shown in table 2. It appears from this analysis that the previously found trend has been present throughout the study: the dose used seems to be sufficient only for those patients who weighed ≤ 70 kg, in other words, those patients whose daily dose was above > 2 mg/kg of body weight.

In view of some data which seem to indicate that

TABLE 2.—Recurrence and death as related to relative dose of levamisole

Patients' weight (kg)	Relative dose of levamisole (mg/kg/day)		Proven relapses/No. treated				Carcinomatous deaths/No. treated			
			Levamisole		Placebo		Levamisole		Placebo	
≤ 60	2.5–3.8 (median: 2.6)	2.1–3.8 (median: 2.3)	2/9 (22.2%)	10/40 (25%)	8/15 (53.3%)	26/52 (50%)	1/9 (11.1%)	6/40 (15%)	7/15 (46.7%)	23/52 (44.2%)
61–70	2.1–2.5 (median: 2.3)		8/31 (25.8%)		18/37 (48.7%)		5/31 (16.1%)		16/37 (43.2%)	
71–80	1.9–2.1 (median: 2.0)	1.1–2.1 (median: 1.9)	18/37 (48.7%)	28/56 (50%)	15/43 (34.9%)	24/62 (38.7%)	14/37 (37.8%)	23/56 (41.1%)	12/43 (27.9%)	20/62 (32.3%)
> 80	1.1–1.8 (median: 1.7)		10/19 (52.6%)		9/19 (47.4%)		9/19 (47.4%)		8/19 (42.1%)	

levamisole might preferentially inhibit metastasis formation (6), the site of the first recurrence was another point of interest. This analysis was restricted to the adequately dosed patients only. Although a trend in favor of levamisole was found for both intrathoracic and distant recurrences, only the latter trend proved to be statistically significant ($P < 0.03$, Fisher test, two-tailed).

Finally, the incidence of side effects was calculated. Gastroenterologic side effects were found in 24% of the levamisole-treated patients versus 22% of the placebo controls. Apart from nervousness and/or sleep disorders (5% with levamisole and 2% with the placebo) and fever and/or flu-like syndrome (4% with levamisole versus 2% with the placebo), other side effects were only occasionally seen. In particular, no instances of allergic agranulocytosis (7) have been found. The small difference in the incidence of side effects between levamisole and the placebo is confirmed if the adverse phenomena are divided into idiosyncratic ones (eg, drug fever, joint aches, mouth ulcers) and all others. This is shown in figure 1.

Actuarial Analysis (8)

For the total population studied, a slight trend which favors levamisole treatment was found regarding recurrence and carcinomatous deaths; this trend reached statistical significance ($P \leq 0.05$, Student's t test, two-tailed) only 18 and 21 months after surgery for the cancer deaths (not shown). However, significant superiority was found when the adequately dosed patients were studied separately (fig 2) whereas no benefit was obtained in the other patients (fig 3). Moreover, the striking difference regarding the effect of levamisole in view of the patients' weight was also found in each of the three cooperating centers.

Further analyses were restricted to the adequately dosed patients. In these patients, levamisole proved significantly better than the placebo in the three histologic groups studied (ie, squamous cell carcinoma, adenocarcinoma, and others plus mixed types). Therefore, the beneficial effect observed in these patients could not be explained by the slight difference between the two medication groups regarding the histologic diagnosis. In contrast to what was expected, not only was the levamisole effect restricted to those patients who had responded only weakly or not at all to the skin test antigens (purified protein derivative and dinitrochlorobenzene), the effect was present to the same extent in both the good and poor responders. Finally, it appeared that the beneficial effect of levamisole was primarily found in those patients who had more advanced cancers before surgery (fig 4). It is not clear from this study whether this is due to the already good prognosis of the control patients with the less advanced tumors or to a reversal by levamisole of the immune suppression associated with the more advanced cancers.

CONCLUSION

Our findings indicate that adjuvant treatment with levamisole in patients with resectable lung cancer ameliorates the prognosis of the patients by prolonging both the disease-free interval and the survival time. This is all the more important because *no* studies of adjuvant chemotherapy of resectable lung cancer have shown any benefit so far (9). However, the dose used in our study appears to be sufficient only for patients weighing ≤ 70 kg and, therefore, in the future, the daily dose should be adapted to the patients' weight or to their body surface area. Thus, an intermittent target dose of 2.5 mg/kg or 100 mg/m²/day, taken in two to three doses, seems recommendable. Skin test reactivity in this study did not allow us to select those patients who benefited from levamisole treatment, although the data in the control group (not shown) do confirm

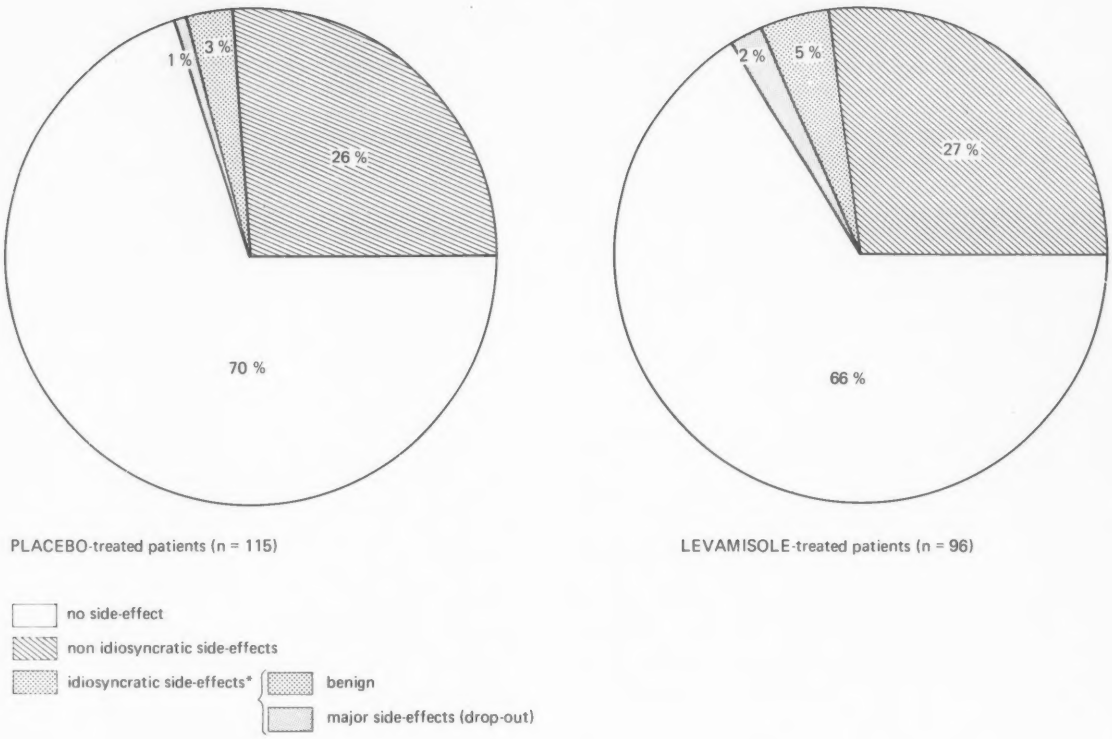

FIGURE 1.—Incidence of side effects.

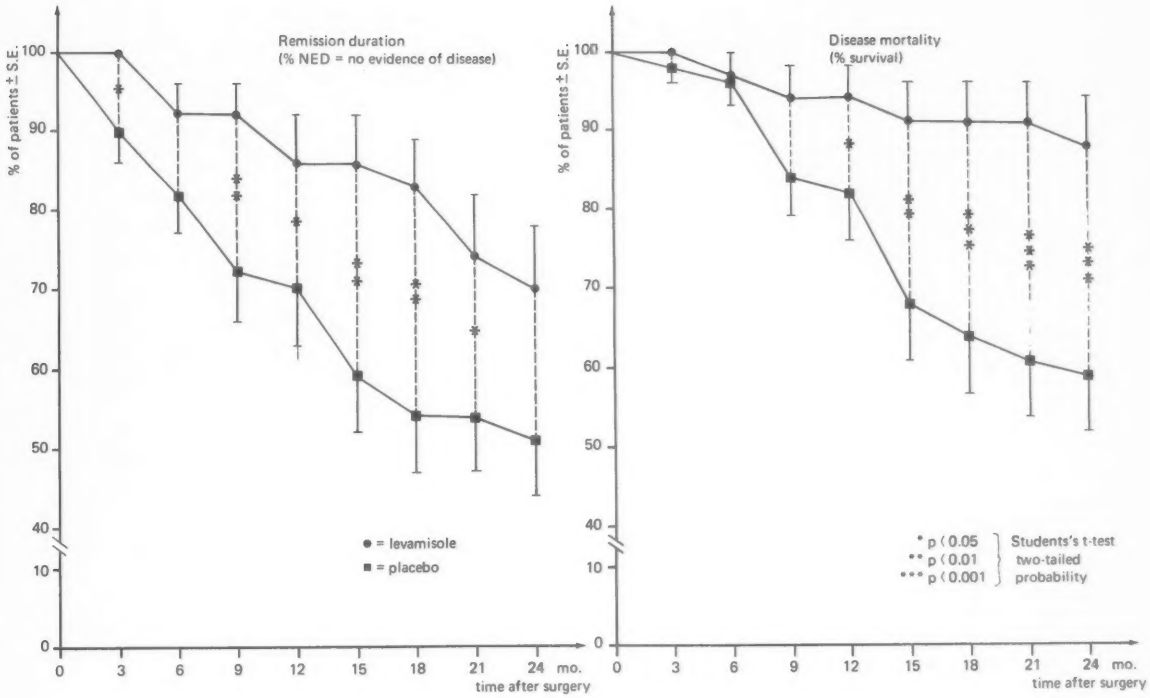

FIGURE 2.—Actuarial analysis: adequately dosed patients only.

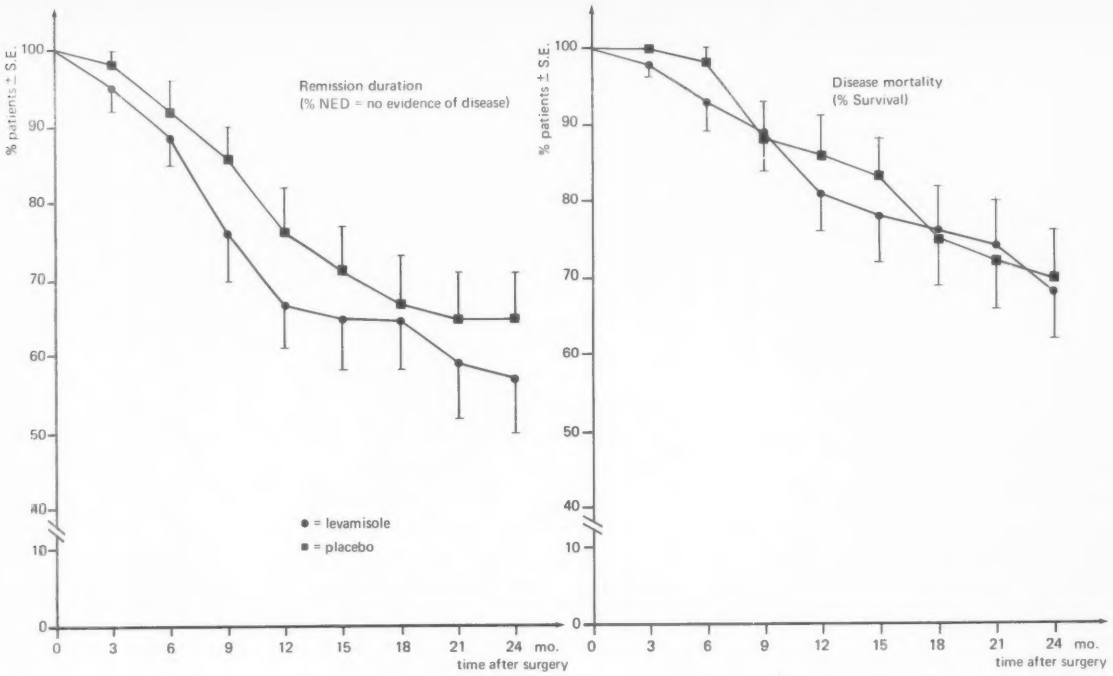

FIGURE 3.—Actuarial analysis: inadequately dosed patients.

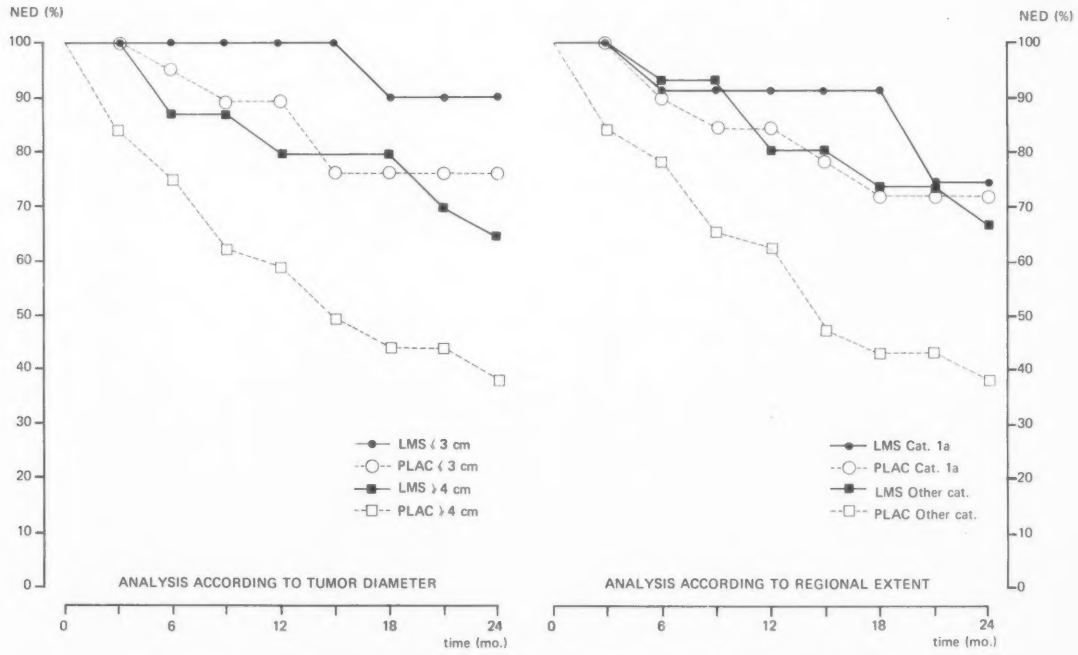

FIGURE 4.—Remission duration as related to tumor extent (adequately dosed patients only).

that these tests have a certain prognostic bearing. These measures are probably not sensitive enough, however, to detect with certainty those patients who have an immune deficiency that can be corrected by levamisole.

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Response of Disseminated Breast Cancer to Combined Modality Treatment With Chemotherapy and Levamisole With or Without Bacillus Calmette-Guérin [1]

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SUMMARY

One hundred and fourteen evaluable patients with measurable metastatic breast cancer were treated with a combination chemoimmunotherapy program (5-fluorouracil, adriamycin, and cyclophosphamide [FAC]-levamisole [LMS]). An additional 117 patients with similar characteristics were treated with the same program with the addition of bacillus Calmette-Guérin (BCG) by scarification (FAC-BCG-LMS). The results were compared to those observed in 44 evaluable patients treated with FAC chemotherapy alone. Overall remission rates were identical in the three studies (72%, 73%, and 72%). The median duration of remission was 9 months for FAC, 13 months for FAC-LMS ($P = 0.07$), and 14 months for FAC-BCG-LMS ($P = 0.05$). The median duration of survival was 17 months for FAC and 28.6 months for FAC-LMS ($P = 0.01$). Although the median survival for the FAC-BCG-LMS group has not been reached, it is significantly superior to FAC alone ($P = 0.009$). These three sequential studies suggest that the addition of nonspecific immunotherapy with LMS or the combination of BCG and LMS prolongs the duration of remission and survival of responding patients treated with chemotherapy. Combination immunotherapy with BCG and LMS did not seem superior to LMS alone in this study.

[Cancer Treat Rep 62:1685–1692, 1978]

Combination chemotherapy has been shown to produce consistently high objective remission rates and overall survival times superior to those achieved by hormonal therapy or single-agent chemotherapy. Over the past 5 years, preliminary reports have appeared suggesting that nonspecific immunotherapy with bacillus Calmette-Guérin (BCG) and *Corynebacterium parvum* is able to prolong the duration of remission and survival of chemotherapy-treated patients (1,2). Levamisole (LMS), an immunorestorative agent, has been reported to prolong disease-free interval and survival of patients with breast cancer following radical radiation therapy (3). We have recently reported our preliminary results with combination chemotherapy and LMS in metastatic breast cancer (4). This paper will update those results and relate our experience with combination chemotherapy, LMS, and BCG in the treatment of stage IV breast cancer patients.

MATERIALS AND METHODS

Two hundred and seventy-four patients with a histologic diagnosis of metastatic breast cancer and

[1] Supported by contract N01-CB-33888 (Division of Cancer Cause and Prevention) and Public Health Service grants CA-05831, CA-09163 (*Dr. Hortobagyi*), CA-71001 (*Dr. Gutterman*), and CA-11430 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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[3] We would like to express our appreciation to Janssen Research and Development, Inc, for kindly providing the levamisole for the study and Audrey Faye Grubbs for her help in the preparation of this manuscript.

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measurable lesions were entered in the programs described in this paper between September 1975 and September 1976. Chemotherapy consisted of 5-fluorouracil (5-FU) (500 mg/m^2 iv on Days 1 and 8), adriamycin (50 mg/m^2 iv on Day 1), and cyclophosphamide (500 mg/m^2 iv on Day 1) over 21-day courses of chemotherapy (FAC). After reaching a total cumulative dose of adriamycin of 450 mg/m^2, this drug was discontinued and maintenance therapy, consisting of 5-FU (500 mg/m^2 orally on Days 1 and 8), methotrexate (30 mg/m^2 im on Days 1 and 8), and cyclophosphamide (500 mg/m^2 orally on Day 2) over 21-day courses of treatment was started. LMS (100 mg/m^2 orally in three divided doses) was administered on Days 9, 10, 13, 14, 17, and 18 of each course of chemoimmunotherapy (FAC-LMS). In the second half of the study, the last 145 patients were treated in addition with BCG (Connaught strain), by scarification, at the dose of 6×10^8 viable units on Days 9, 13, and 17 of each course of chemotherapy (FAC-BCG-LMS). Fifteen patients in the FAC-LMS group and 28 in the FAC-BCG-LMS group were excluded from analysis because of inadequate followup (lost to followup) or major protocol deviations.

The results of both programs were compared to a group of 44 patients with metastatic breast cancer treated with FAC chemotherapy alone between May 1973 and April 1974. This control group has been described before (1). Criteria for eligibility for all three studies were identical and consisted of histologic evidence of breast cancer, objective evidence of progressive disease, and clearly measurable lesions. Patients were excluded if central nervous system involvement was present, if there was evidence of resistance to any of the agents included in this treatment program, or if associated medical conditions (ie, congestive heart failure) precluded the use of any of the agents included in the protocol.

Baseline evaluation included a complete history and physical examination, cbc count, chemical survey (SMA-100), CEA, electrocardiogram, chest roentgenogram, bone survey, bone scan, liver scan, and other tests deemed necessary by each investigator. While tumor measurements were obtained as frequently as possible, a complete restaging workup was performed every 3–4 months. Definitions of response were essentially identical to those described by the UICC (5). Duration of response was measured from the start of treatment and from the time an objective regression is measured until relapse.

The Kaplan and Meier method was used to calculate and plot remission and survival curves and the generalized Wilcoxon test with a one-tailed analysis was utilized to test the differences between remission and survival curves (6,7). The chi-square test was used to assess the comparability of groups by prognostic factors.

RESULTS

There were 114 evaluable patients treated with the FAC-LMS program and 117 patients treated with the FAC-BCG-LMS program. Forty-four patients treated with FAC chemotherapy alone represented the control group. Table 1 shows the pretreatment patient characteristics of all three treatment groups. These include characteristics such as age, menopausal status, and disease-free interval, traditionally thought of as having prognostic importance. No significant differences were noted between the three groups. The distribution of dominant sites of disease and disease extent was also similar in the three groups of patients.

Table 2 shows the overall response rates to all three treatment programs. Depending on the dominant site of the disease, objective responses were achieved at different periods of time following the beginning of chemotherapy. Thus, the median time to achieve a partial remission was 9 weeks for soft tissue and visceral disease and 20 weeks for osseous disease. Consequently, the response rates shown in table 2 are cumulative, measured at 18 months following initiation of chemotherapy. As shown in this table, the overall response rates and the complete response rates were virtually identical in all three studies. This is in keeping with our previously published results.

Figure 1 shows the duration of remission (complete remission + partial remission) for all three treatment regimens. The median duration of response for the FAC program was 9 months while it was 13 months for FAC-LMS and 14 months for FAC-BCG-LMS. Although there is no statistical difference between these latter two groups, both are significantly superior to the FAC chemotherapy-alone group ($P = 0.07$ and $P = 0.05$ for FAC-LMS and FAC-BCG-LMS respectively).

The length of survival of responding patients was 17 months for the FAC group and 28.6 months for the FAC-LMS group; median survival values have not been reached for the FAC-BCG-LMS group (fig 2). Again, while the latter two groups are not statistically different, they are both significantly superior to the FAC-alone program ($P = 0.01$ and $P = 0.009$ for FAC-LMS and FAC-BCG-LMS respectively).

Figure 3 shows the survival of all treated patients, regardless of response. The median survival was 15 months for the FAC group, 23.3 months for the FAC-LMS group, and 21.4 months for the FAC-BCG-LMS group. While these latter two groups

TABLE 1.—Pretreatment population characteristics

	Program		
	FAC	FAC-LMS	FAC-BCG-LMS
No. of evaluable patients	44	114	117
Median age in years (range)	51 (29–67)	53 (23–73)	51 (26–71)
Race (%)			
White	88	88	87
Black	5	5	3
Latin	7	7	10
Median disease-free interval (mos) (range)	15 (0–104)	15 (0–186)	18 (0–276)
Menopausal status (%)			
Pre	25	11	14
Postoophorectomy	9	27	25
Post	66	62	61
Prior therapy (%)			
Hormonal	79	64	48
Chemotherapy	7	10	10
Dominant site (%)			
Soft tissue	4.5	4	6
Osseous	18	30	32
Visceral	77	66	61
No. of disease sites (%)			
1–2	59	54	71
> 3	41	46	29

TABLE 2.—Response rates

	Program		
	FAC: No. of patients (%)	FAC-LMS: No. of patients (%)	FAC-BCG-LMS: No. of patients (%)
No. of evaluable patients	44	114	117
Complete remission	6 (14)	15 (13)	19 (16)
Partial remission	26 (59)	67 (59)	66 (56)
Stable	12 (27)	26 (23)	27 (23)
Progressive	—	6 (5)	5 (4)
Overall remission	32 (73)	82 (72)	85 (72)

survived longer than the chemotherapy-alone patients, this difference only borders significance (P = 0.09).

TOXICITY

Tolerance and toxicity of the FAC chemotherapy program have been reported before (8), and they were similar in the three treatment groups (table 3). Nausea, vomiting, and alopecia were the most common side effects, while a number of other symptoms occurred in a small number of patients. The severity of these symptoms varied from none to severe, but in most patients they were mild to moderate. Unquestionably, the dose-limiting toxicity of the program was myelosuppression. Compar-

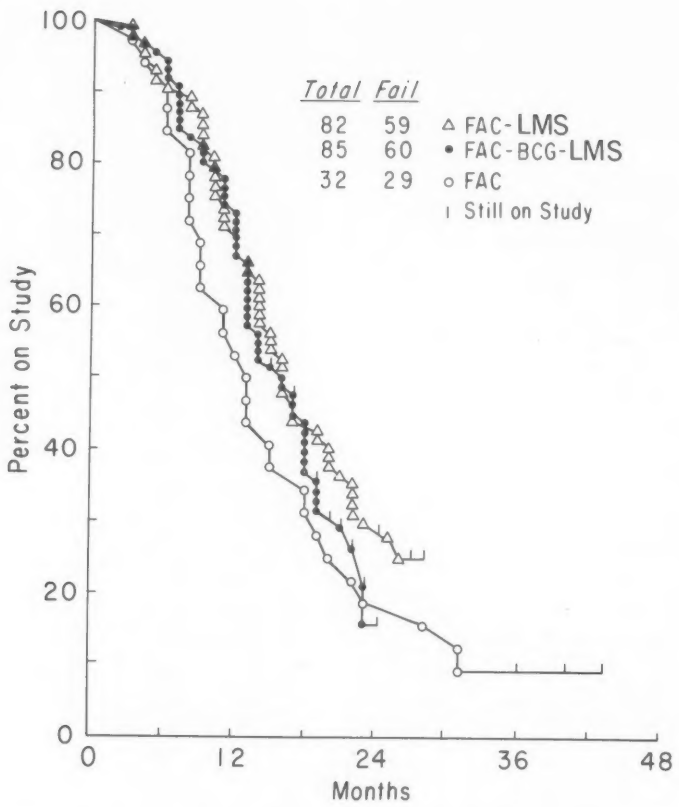

FIGURE 1.—Duration of remission in patients receiving FAC-LMS, FAC-BCG-LMS, and FAC.

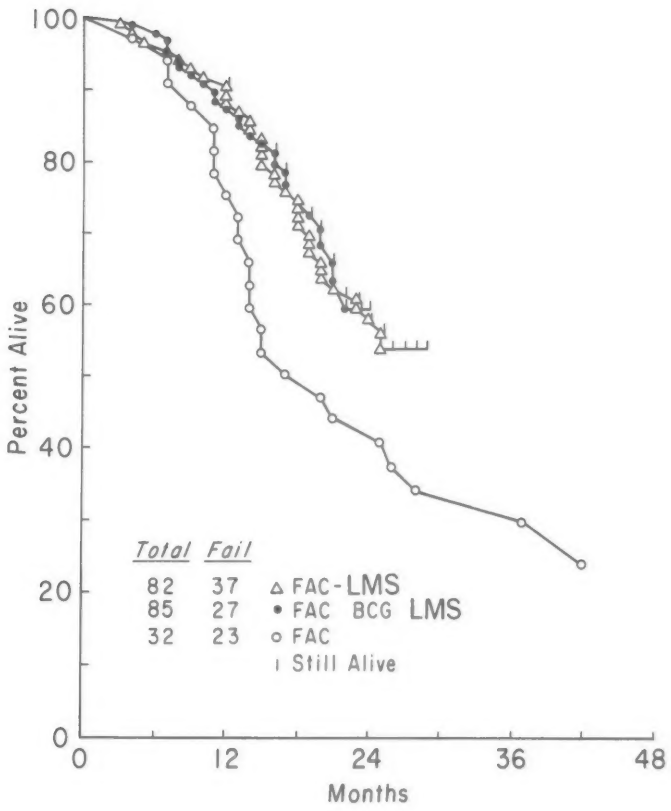

FIGURE 2.—Length of survival of responding patients.

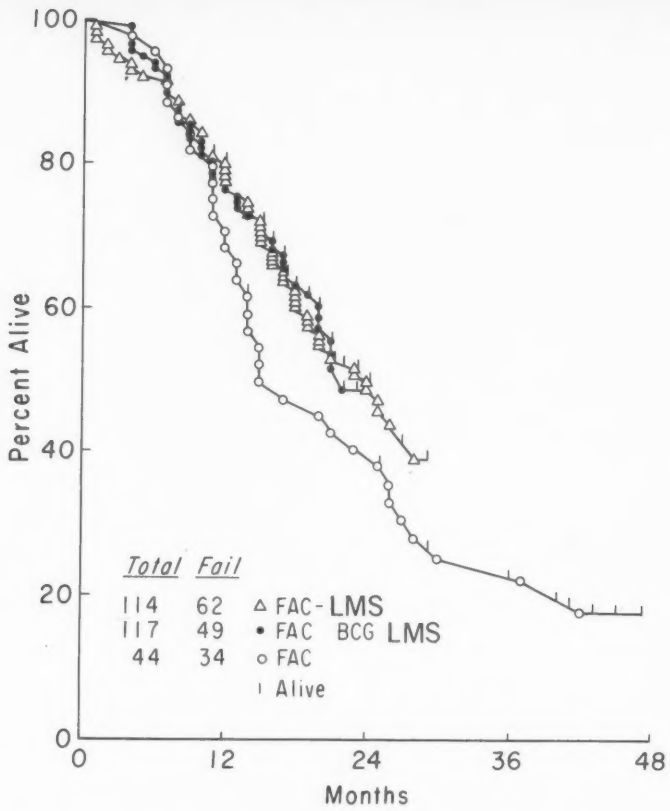

FIGURE 3.—Survival of all treated patients.

TABLE 3.—Nonhematologic toxicity

Symptom	% patients
Nausea	77
Vomiting	74
Alopecia	30
Mucositis	14
Fever of unknown origin	14
Minor infections	12
Weakness	9
Weight loss > 5 lbs	8
Anorexia	8
Diarrhea	7
Sepsis	7
Phlebitis	6
Congestive heart failure	3
Skin pigmentation	2
Adriamycin infiltration	2
Septic death	< 1
Hematuria	1

ative myelosuppression in the programs is shown in table 4. No significant differences in nadir of leukopenia or thrombocytopenia were observed in the treated groups. By the same token, the percent of protocol doses of drugs administered was similar for all three groups of patients.

While the incidence of minor infections was approximately 14%, major infections were uncommon (7%), and only one septic death was observed in the FAC-LMS study. This is not different from our previous experience with chemotherapy alone. Bleeding related to thrombocytopenia was not observed.

Table 5 shows a list of those symptoms that could be attributed to the administration of LMS. This drug was extremely well-tolerated and the incidence of side effects was very small. Nausea and vomiting were the most commonly observed symptoms while a variety of other subjective and objective disturb-

TABLE 4.—Hematologic toxicity

	FAC		FAC-LMS	
	1	6	1	6
	(Treatment cycle No.)			
Median lowest wbc count (cells × 10^3/mm^3) (range)	2.4 (0.1– 5.0)	2.7 (0.8–6.2)	2.0 (0.4–5.2)	2.2 (1.0–6.1)
Median lowest polymorphonuclear neutrophil count (cells × 10^3/mm^3) (range)	0. 86 (0–3.8)	1.4 (0.2–3.1)	0.8 (0–3.4)	1.0 (0–3.1)
Median lowest platelet count (cells × 10^3/mm^3) (range)	180 (41–345)	152 (65–265)	209 (10–574)	160 (55–356)
% protocol dose				
Median	100	80	100	80
Mean	93	81	95	86

TABLE 5.—LMS toxicity*

Symptom	%
Gastrointestinal	
Nausea	21
Fever	13
Vomiting	12
Bitter taste	5
Chills	3
Anorexia	3
Diarrhea	1
Hunger	1
Halitosis	1
Mucositis	1
Autonomic	
Tachycardia	2
Diaphoresis	2
Hot flashes	1
Facial edema	1
Chest tightness	1
Urinary frequency	1
Tearing	1
Neurologic	
Hyperosmia	4
Headache	4
Lethargy	4
Emotional lability	3
Depression	2
Irritability	2
Dizziness	2
Blurry vision	2
Hyperacusia	1
Red and green lights	1
Syncope	1
Depersonalization	1
Nightmares	1
Miscellaneous	
Malaise	6
Weakness	4
Skin rash	2
Neutropenia	1

*Dose decreased in 7% of patients. Drug discontinued in 5% of patients.

ances were reported by a small number of patients. A dose reduction of LMS had to be done in 7% of the patients to decrease or eliminate excessive gastrointestinal or neurologic side effects; only 5% of the patients had to discontinue LMS for reasons related to toxicity.

The combination of BCG and LMS was very well-tolerated and no added side effects were observed. Local symptoms attributable to scarification were observed in some patients while a maculopapular systemic rash was seen in two. No disseminated BCG disease was seen in this or any of our previous studies.

DISCUSSION

These three studies demonstrated that combination chemotherapy with FAC is an effective form of systemic treatment, achieving a high remission rate and improved survival. Furthermore, they showed that the addition of nonspecific immunotherapy with LMS or the combination of BCG and LMS prolongs the duration of chemotherapy-produced remissions and the survival of patients who achieve such remissions. In this report, as in previous reports, those patients who did not achieve an objective remission derived no benefit from the administration of nonspecific immunotherapy. The results of the FAC chemotherapy-alone regimen are similar to those obtained by the use of adriamycin and cyclophosphamide (9) or by other adriamycin-containing regimens (10,11).

As in any comparative study, minor maldistribution of prognostic factors can be expected. In this study, the FAC control group had a somewhat higher percentage of patients with predominately visceral disease which might have biased the study in favor of the chemoimmunotherapy groups. On

the other hand, this difference is so small that it could not possibly explain the sizable differences in survival. Since the study group was treated > 1 year after the control group was accrued, improvement in supportive care and antineoplastic treatment after relapse could account for some of the differences observed. No differences were observed in the two groups as related to prechemotherapy prognostic factors or locoregional and systemic treatment.

Nonspecific immunotherapy with BCG has been shown to prolong remission and survival in a number of malignant diseases (12–15), including breast cancer. LMS, while prolonging survival to an extent similar to that observed with BCG (4), is thought to act by a different mechanism of action. We hypothesized that the combination of an immunorestorative agent, such as LMS, and an immunopotentiating agent, such as BCG, might produce additive or synergistic effects in the immune system. However, this was not borne out in the second part of our study, and the results obtained with the combination of both immunomodulating agents were not superior to those obtained with either agent alone.

The treatment programs described in this paper have been very well-tolerated. Although some side effects were experienced by the majority of patients, life-threatening toxicity is distinctly unusual.

A large number of chemotherapy and chemoimmunotherapy trials including LMS are currently being conducted throughout the world. Recently, two independent investigators reported results similar to ours, using similar chemotherapy and immunotherapy programs (16). It is hoped that longer followup will confirm these results and clearly establish the role of LMS in the treatment of advanced breast cancer.

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Levamisole as an Immunoadjuvant: Phase I Study and Application in Breast Cancer [1]

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SUMMARY

A phase I study of levamisole in patients with cancer was undertaken to determine its therapeutic dose range, side effects, limiting toxicity, and influence on immune function. Simultaneously, a group of patients receiving adjuvant combination chemotherapy for carcinoma of the breast were entered in a randomized study to observe whether levamisole was capable of overcoming the immunodepressive effects of cytotoxic drugs. In the phase I trial the doses used ranged from 65 to 410 mg/m². The maximum tolerated dose was 358 mg/m². Side effects were generally low in intensity and related to the gastrointestinal and central nervous systems; the limiting toxic effect was intractable nausea. Significant changes in immune test values were noted in both acute and chronic trials. Alterations in immunoglobulin levels, complement values, and numbers and types of lymphocytes and mitogenic responses to concanavalin A as well as specific mitogens were observed. In the randomized trial there was no difference between the chemoimmunotherapy and chemotherapy-alone groups.

[Cancer Treat Rep 62:1693–1701, 1978]

Levamisole is a drug which has been reported to have a broad spectrum of immunoadjuvant effects. It is said to augment humoral immunity (1,2), cell-dependent immunity (3,4), and macrophage function (5), including phagocytosis (6). Furthermore, it seems to be a versatile therapeutic agent having been employed in the treatment of warts (7), atopic dermatitis,[3] aphthous stomatitis (8), herpes simplex (9), rheumatoid arthritis (10), lupus erythematosus (11), nematodal infections of man and cattle (12,13), bovine mastitis (14), and carcinoma of the lung and breast (15,16). Its best-established therapeutic role is as an anthelmintic agent in the treatment of infections by nematodes.

In the field of cancer immunotherapy, levamisole is considered by some (15) to serve not as an immunopotentiator which increases immune function in treated patients, but as an immunomodulator which restores immune responsiveness in those patients with previously depressed immune reactivity. Despite its wide use, neither the therapeutic index of levamisole nor its toxicity in man at dose levels > 70 mg/m² has been fully documented.

In this paper the results of a phase I study of levamisole in patients with cancer are reported. This study was undertaken to determine (*a*) the therapeutic dose range of levamisole, including its maximum tolerated dose (MTD), (*b*) the side effects at clinically tolerated dose levels, (*c*) the limiting toxicity at maximum doses, and (*d*) any immune changes that occurred during the phase I trials. Also presented are preliminary immune data available from an ongoing study of adjuvant therapy for the prevention of recurrent breast carcinoma. In this investigation the patients received combination chemotherapy alone or chemoimmunotherapy which

[1] Supported by Public Health Service grant CA-05826 and contract CB-53970 (Division of Cancer Biology and Diagnosis) from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, and grant IM-26A from the American Cancer Society.

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included levamisole. The intention was to learn whether levamisole is capable of overcoming the immunodepression which chemotherapy is thought to produce.

MATERIALS AND METHODS

The phase I study design is presented in figure 1. Drug effects were observed after a short-term acute trial and during long-term drug administration in a chronic trial. Each patient received the same dose during both phases of the study. The dose for the first three patients was 150 mg daily for each treatment day. After three patients had been entered at this level the next three patients were given 300 mg/day. The dose was further escalated by 150 mg in each successive group of three patients until a level was reached that produced sufficient toxic reactions to cause patients to refuse to continue their trials. Additional patients were then entered at lower dose levels with one patient added to each group, escalating as before to the MTD.

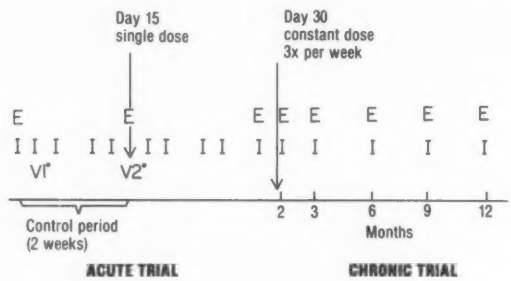
The acute-dose trial of levamisole was conducted using two schedules of drug administration. The first 16 patients received a single dose as shown in figure 1 and were observed for 2 weeks before and 2 weeks after receiving levamisole. When preliminary data analysis showed no effect on immune function, subsequent patients entered were given levamisole daily for 7 days. Observations were made 2 weeks before giving the drug and then from Day 1 to 14 after starting treatment. In the chronic-dose trial patients received medication every Monday, Wednesday, and Friday.

History, physical examination, and laboratory tests to determine toxicity, including blood cell and differential counts, SMA-12, and urinalysis, were performed at the points marked "E" in figure 1: ie, prior to entry into the protocol, about 1 hour after the initial dose, at the end of the first month, at the second and third months, and every 3 months thereafter until the drug was discontinued or 2 years had passed.

For the first 16 patients immune parameters were measured at all points marked "I." Tests were performed before patient entry, four times, ie, twice a week for 2 weeks before the first dose, and then four times in the next 2 weeks. Observations were then made monthly for 3 months and continued thereafter every 3 months. In the acute trial, patients received a vaccine at points marked "V_1" and "V_2" The vaccines used were for typhoid fever and influenza. For the initial vaccination these two vaccines were alternately used. Then at the time of the second vaccination the remaining vaccine was administered. The immune parameters measured are listed in figure 2. Data relating to skin tests and antibody responses to vaccination have not been fully analyzed and are, therefore, not presented in this paper.

Quantitation of serum immunoglobulins (IgG, IgA, and IgM) was performed by single-radial immunodiffusion using commercial plates (17).

T rosettes in the blood were enumerated by the method of Jondal et al (18) with minor modifications. Human AB serum (20%), absorbed three times with sheep red blood cells, was included in the lymphocyte suspension for the determination of the number of spontaneous rosette-forming lymphocytes with sheep erythrocytes. B lymphocytes were determined by the number of C_3-receptor lymphocytes (EAC-rosettes) and by the number of lymphocytes positive for surface immunoglobulins by direct immunofluorescence using a fluoresceinated polyvalent anti-immunoglobulin antiserum (19). The



E Extent of disease and toxicologic tests
I Immunologic evaluation
V1*,V2* Vaccine administration (See worksheet)

Dose fixed for each patient, escalates after each group of 3 patients

FIGURE 1.—Phase I study design.

Humoral Immunity
- Immunoglobulins: IgA, IgG, IgM
- B cells (%)
- Complement

Cell-Mediated Immunity
- Lymphocyte count
- T cells (%)
- In vitro mitogen response
  - Nonspecific and specific stimuli: phytohemagglutinin, Con A, pokeweed mitogen, *C. albicans*, *E. coli*, *S. aureus*, tuberculin, streptokinase/streptodornase, mumps, and mixed bacterial vaccine

Reticuloendothelial
- NBT
  - Resting
  - Lipopolysaccharide-stimulated

FIGURE 2.—Phase I immunotherapy trial: immune parameters.

method for performing EAC-rosettes was a modification of the technique of Bianco et al (20) using human A erythrocytes and fresh mouse serum as a source of complement. The ingestion of latex particles was used as a marker for phagocytic monocytes (21).

Lymphocytes were isolated from heparinized blood on Ficoll-Isopaque density gradients. After washing and resuspension in RPMI-1640 supplemented with glutamine, penicillin, streptomycin, and 15% pooled normal human serum, 0.2 ml of mononuclear cell suspension containing 1×10^5 lymphocytes was incubated in microtiter plates. The transformation of lymphocytes was measured by incorporation of ^{14}C-thymidine. All individual cultures were performed in triplicate. The stimulation with nonspecific mitogens was performed so as to derive a dose-response curve using six different concentrations of phytohemagglutinin-P, four different concentrations of concanavalin A (Con A), and three different concentrations of pokeweed mitogen. The standard panel of microbial antigens included *Staphylococcus aureus*, *Candida albicans*, *Escherichia coli*, streptokinase-streptodornase, two highly purified protein derivatives of *Mycobacterium*, and mumps virus. A dose curve using different concentrations was performed for each antigen.

Buffers for complement and C-component assays were determined as previously described (22).

To assay phagocyte function the histochemical nitroblue tetrazolium (NBT) test was used. This screening test measures the capacity of stimulated phagocytes to reduce NBT. The results reflect respiratory burst activity and indicate superoxide production. In the assay, heparinized whole blood is used. One aliquot is employed to determine the resting value and the other to determine the endotoxin stimulation. After incubation with 0.1% of NBT, cover slip smears were prepared and stained, and the percent of NBT-positive cells was counted under oil immersion. Total wbc and differential counts were also done (23).

The adjuvant therapy study to prevent recurrent carcinoma of the breast calls for randomization of patients to receive either combination chemotherapy with cyclophosphamide, methotrexate, and 5-fluorouracil (CMF), or CMF plus levamisole. Treatment is continued for 2 years. Levamisole is given on 3 consecutive days every 2 weeks.

RESULTS

There were 46 patients entered in the acute phase I trial and 40 in the chronic trial. Table 1 shows the distribution by sex and age of those patients studied. There was a small preponderance of males. The median age in both trials was 55 years.

The diagnoses for the patient population treated are listed in table 2. Nearly all of these patients had distant metastases. Since patients with melanoma are among those most frequently referred for immunotherapy, this was the largest single diagnostic category. In addition to those patients entered for therapy for their malignant conditions, three patients with lymphoma were referred for treatment of other diseases which were said to respond to levamisole: one with condylomata lata of the vulva and two with warts.

The total doses administered are shown in table 3. The dose range was 65–410 mg/m^2. Most patients refused to take doses > 300 mg/m^2 on more than one occasion.

Since doses were not deliberately increased to maximum-tolerated levels in each patient studied, it was not possible to give a precise value for the mean MTD. To overcome this problem, an additional 12 patients were given levamisole in an escalating-dose study to be reported in detail elsewhere. The initial total dose was 150 mg orally on Day 1 of these trials. Approximately every other day the dose was increased by one 150-mg tablet until the MTD for each patient was reached. At first, all tablets were taken together; however, the patients then complained of gastrointestinal distress and the doses were divided and patients were given the drug in three equal doses daily. If nausea was the major complaint, an anti-emetic agent was given to alleviate the symptoms. With this deliberate effort to reach the MTD, the maximum quantity of drug accepted by patients ranged from 300 to 900 mg (total dose) or from 189.9 to 542.2 mg/m^2. The mean MTD was 358 mg/m^2.

Side effects of levamisole given in the acute and

TABLE 1.—Phase I levamisole study: patients entered in trials by age and sex

Trial	Route	No. of patients			Median age (yrs)
		Entered	Male	Female	
Acute	Oral	46	25	21	55
Chronic	Oral	40	24	16	55

TABLE 2.—Phase I levamisole study: diagnoses of patient entered

Diagnosis	No. of patients	
	Acute	Chronic
Bronchial carcinoid	1	1
Carcinoma		
Bile duct	1	1
Breast	5	2
Breast and bladder		1
Breast, cervix, and ovary	1	1
Colon		1
Esophagus	1	1
Lung	4	3
Ovary	1	1
Pancreas		1
Parotid	1	1
Prostate	1	2
Rectum		1
Stomach		1
Thyroid	6	
Tongue	2	2
Condyloma of vulva	1	
Fibrous histiocytoma	2	1
Hodgkin's disease	2	2
Kaposi's sarcoma	1	1
Lymphoma	3	3
Melanoma	10	8
Myeloma	1	1
Renal cell sarcoma	2	2
Warts		2
Total	46	40

TABLE 3.—Phase I levamisole study: dose range by trial

Dose (mg)	No. of patients	
	Acute	Chronic
150	26	21
300	9	11
450	6	9
600	4	2
750	1	1
Total	46	44

chronic trials are shown in table 4. Most complaints were related to the gastrointestinal and central nervous systems. As the doses increased, no new toxic symptoms appeared. Instead, previously observed symptoms occurred with higher frequency. Nausea was consistently the dose-limiting toxic effect.

An analysis of the results of serial immune parameter measurements over 2 weeks in 34 as yet untreated patients presenting for phase I immunotherapy trials has shown no significant changes with time. Data were analyzed both by the one-way analysis of variance and by the Kruskal-Wallis test.

As mentioned in the Materials and Methods section, the acute-dose trial of levamisole was conducted using two schedules of drug administration. The first 16 patients received a single dose and subsequent patients were treated for 7 days. The significant laboratory test findings for those patients given a single dose, relating both to toxic and immune effects, are given in table 5. No changes were seen in tests reflecting bone marrow, liver, and kidney function. Noted was a minor increase in IgM levels during the second week following treatment and an increase in the relative number of B cells within 2–3 days of levamisole ingestion. Both these changes were statistically significant with $P < 0.05$ by the paired t test.

In those patients given levamisole for 7 days (table 6) there was a consistent fall in hemoglobin and uric acid values. There was no change in IgM levels or in the relative numbers of B cells in the peripheral blood. However, there were significant early elevations in IgG and total complement levels.

Combining these data, the gradual fall in hemoglobin, and to a lesser extent the fall of uric acid levels in the peripheral blood following treatment, was confirmed. As seen in table 7, the combined data on acute trials show four significant changes in immune parameters. These are (*a*) an increase in IgG levels by 2–3 days persisting to 8–14 days, (*b*) a rise in the relative number of circulating B cells, (*c*) an increase in total complement values, and (*d*) a decrease in lymphocyte blastogenesis response to Con A.

The significant laboratory test changes for chronic-dose levamisole are shown in table 8. The wbc count decreased transiently but significantly at 3 months. Lower uric acid values were evident at 3, 6, and 9 months. Serum concentrations of 5′-nucleotidase were also lower at 3 and 6 months. Significant immune parameter changes included a modest decrease in IgM levels when measured at 6–9 months. The relative numbers of circulating lymphocytes decreased significantly to a low point by 2 months but rose soon thereafter to normal values. Lymphocyte stimulation induced by Con A remained fairly constant but increased transiently at 9 months. There were consistent rises in the blastogenic response to *S. aureus* and *E. coli*.

In the breast cancer adjuvant chemotherapy study, which is still underway, only a portion of the patients have had serial measurements of immune parameters. These include 22 patients receiving

TABLE 4.—Phase I levamisole toxicity

Toxic effect	No. of patients receiving— 150 mg (28)*	300 mg (14)	450 mg (7)	600 mg (4)	750 mg (1)
Nausea	3	7	4	3	1
Bitter taste	3	3			
Lethargy	5	2	2	2	
Cramps	3	4	2	2	
Diarrhea	2	2	2	2	
Insomnia	2	3	2		
Headache	1	1		2	
Emesis	3	1	3	2	1
Dizziness	2	5	3	3	
Eructation	3	3	2		
Fever	1				
Blurred vision		1	1		
Thirst		1			
Photophobia		1			
Numbness		1	1		
Confusion		1	1		1
Rash	2	2			
Syncope	1	1			
Itching	2	3			
Queasiness	4	5	1		
Lightheadedness	3	3	2		
Restlessness	1	1			
Anorexia	1	1			
Gas		2			
Increase in frequency of bowel movements	1	2	1		

*Nos. in parentheses = total No. of patients treated.

TABLE 5.—Significant paired t tests for single-dose levamisole

Test	Mean pretherapy value*	Mean later value	Mean change	Later time point (days)
IgM	172 (13)	188†	16	8–14
B cells	10 (6)	15	5	2–3

*Nos. in parentheses = sample sizes.
†$P < 0.05$, 2-sided paired t test.

TABLE 6.—Significant paired *t* tests for 7-day levamisole trial

Test	Mean pretherapy value*	Mean later value	Mean change	Later time point (days)
IgG	1283 (14)	1428†	145	2–3
IgG	1283 (14)	1484†	201	4–7
Total complement	116 (9)	132‡	16	1
Hemoglobin	13.3 (9)	12.6†	– 0.7	1
Hemoglobin	12.4 (13)	12.0‡	– 0.4	2–3
Hemoglobin	12.8 (14)	11.8†	– 1.0	4–7
Hemoglobin	12.8 (14)	11.5†	– 1.3	8–14
Uric acid	5.5 (10)	4.7†	– 0.8	4–7

*Nos. in parentheses = sample sizes.
†$P < 0.01$, 2-sided paired *t* test.
‡$P < 0.05$, 2-sided paired *t* test.

TABLE 7.—Significant paired *t* tests for acute-dose levamisole

Test	Mean pretherapy value*	Mean later value	Mean change	Later time point (days)
IgG	1224 (21)	1358†	134	2–3
IgG	1239 (29)	1343‡	104	4–7
IgG	1256 (27)	1387‡	131	8–14
Total complement	116 (9)	132‡	16	1
C1q	27.8 (21)	25.9‡	– 1.9	2–3
B cells	10 (6)	15‡	5	2–3
Con A	11,246 (25)	8914‡	– 2332	8–14
Hemoglobin	13.3 (9)	12.6†	– 0.7	1
Hemoglobin	12.5 (16)	11.9†	– 0.6	2–3
Hemoglobin	12.8 (20)	11.9†	– 0.9	4–7
Hemoglobin	12.9 (24)	11.8†	– 1.1	8–14
Uric acid	5.5 (11)	4.7†	– 0.8	4–7

*Nos. in parentheses = sample sizes.
†$P < 0.01$, 2-sided paired *t* test.
‡$P < 0.05$, 2-sided paired *t* test.

chemotherapy (CMF) alone and 20 patients receiving chemotherapy (CMF) plus levamisole. An analysis of the available data is provided in table 9. The question asked of these data was whether the proportion of values showing increases or decreases is different in the two groups. Fisher's exact test was used to compare proportions. There were no statistically significant differences with the exception of an increase in the blastogenic response to phytohemagglutinin in the levamisole-treated group at 8 months.

DISCUSSION

The standard dose of levamisole used in nearly all clinical trials to date has been 150 mg/day. This dose was arbitrarily selected by Renoux[4] who reported that it was sufficient to produce immunopotentiation. More recently, Amery (15) suggested that doses < 2.5 mg/kg are ineffective. This is based on his analysis of a prospective study in patients with

[4]Renoux G. Personal communication.

TABLE 8.—Significant paired *t* tests for chronic phase I levamisole trial

Test	Mean pretherapy value*	Mean later value	Mean change	Later time point (mos)
IgG	1180 (20)	1372†	192	2
IgM	157 (12)	109†	−48	6
IgM	82 (6)	63†	−19	9
Lymphocytes	24 (13)	17†	−7	2
Con A	10,043 (8)	13,175‡	3132	9
E. coli	696 (5)	4902†	4206	9
S. aureus	2078 (10)	6552†	4474	2
S. aureus	2478 (7)	5731†	3253	9
S. aureus	2911 (5)	5717†	2806	12
Wbc count	6.3 (10)	4.5†	−1.8	3
Uric acid	6.6 (12)	5.9†	−0.7	3
Uric acid	6.4 (10)	5.7†	−0.7	6
Uric acid	6.7 (7)	6.0†	−0.7	9
5′-nucleotidase	10.9 (8)	7.8†	−3.1	3
5′-nucleotidase	8.2 (7)	5.6†	−2.6	6

*Nos. in parentheses = sample sizes.
†$P < 0.05$, 2-sided paired *t* test.
‡$P < 0.01$, 2-sided paired *t* test.

TABLE 9.—Breast carcinoma adjuvant study: proportion of patients with increasing immune parameters

Parameter	Treatment	No. of patients with increasing parameters/No. studied							
		2 mos		4 mos		8 mos		12 mos	
Phytohemagglutinin	CMF	0/3		3/17	18%	0/13		0/12	
	CMF + levamisole	1/3	33% (1.0)*	5/16	31% (0.44)	7/17	41% (0.01)	1/15	7% (0.36)
Con A	CMF	2/3	67%	7/17	41%	4/13	31%	4/12	33%
	CMF + levamisole	2/3	67% (1.0)	8/16	50% (0.73)	10/17	59% (0.16)	3/14	21% (0.67)
Pokeweed mitogen	CMF	2/3	67%	6/16	38%	7/13	54%	5/12	42%
	CMF + levamisole	2/3	67% (1.0)	11/16	69% (0.16)	14/17	82% (0.12)	7/14	50% (0.71)
C. albicans	CMF	1/3	33%	8/15	53%	4/12	33%	2/9	22%
	CMF + levamisole	2/3	67% (1.0)	9/15	60% (1.0)	9/13	69% (0.12)	3/10	30% (1.0)
E. coli	CMF	2/2	100%	3/15	20%	6/11	55%	6/11	55%
	CMF + levamisole	0/2	(0.33)	4/15	27% (1.0)	7/13	54% (1.0)	3/12	25% (0.21)
S. aureus	CMF	1/3	33%	5/14	36%	3/12	25%	4/10	40%
	CMF + levamisole	2/3	67% (1.0)	4/15	27% (0.70)	5/14	36% (0.68)	6/13	46% (1.0)
Purified protein derivative	CMF	2/3	67%	6/15	40%	3/11	27%	2/10	20%
	CMF + levamisole	2/3	67% (1.0)	7/15	47% (1.0)	3/14	21% (1.0)	4/13	31% (0.66)
Absolute lymphocytes	CMF	0/2		1/12	8%	3/11	27%	1/9	11%
	CMF + levamisole	1/4	25% (1.0)	1/16	6% (1.0)	4/16	25% (1.0)	2/13	15% (1.0)

*Nos. in parentheses = *P* value.

carcinoma of the lung who received levamisole to prevent recurrence following radiation. The findings reported here support the conclusions of the manufacturer that doses of 150 mg or 2.5 mg/kg are generally safe, well-tolerated, and produce minimal toxicity.

The MTD of levamisole has not previously been rigorously determined. In the current phase I study an escalating-dose scheme of drug administration was at first avoided because of animal data indicating that the limiting toxic effects consisted of convulsions followed by death. The refusal of many patients to take doses > 300 mg/m² and the knowledge that doses > 500 mg/m² did not produce serious toxicity prompted us to complete a formal escalating-dose study to be certain that patient refusal to take larger doses was due to limits of tolerance rather than poor cooperation. A mean MTD of approximately 350 mg/m² is now established as well as the limiting toxic effect in man: intractable nausea.

Most of the toxicity of levamisole is low in intensity so that the true incidence of toxic symptoms can only be elicited by careful questioning of patients. The most frequent clinical manifestations of toxicity are related to the gastrointestinal and central nervous systems and this is confirmed by most other studies with this agent (15).[5] This phase I study provides the additional information that at higher doses no new toxic symptoms or signs appear but instead those already known occur with a higher incidence.

There were significant changes in immune function observed in this population. These were low in magnitude, however. The heterogeneity of the patients treated in regard to diagnosis, extent of disease, and treatment history, as well as the lack of controls which is characteristic of a phase I study design, prevents the conclusion that the immune changes seen are due to levamisole. The immune data, however, provide an appropriate base for planning specific studies to observe the influence of levamisole on the various immune functions.

If levamisole were indeed an immunomodulator it might have been expected to correct the immunodepression produced in patients receiving combination chemotherapy. It is disappointing that no such restoration of immune reactivity was seen in breast cancer patients receiving adjuvant chemotherapy who were given levamisole. It may be that such an effect would have been evident if the treatment had been less intensive.

In conclusion, levamisole appears to be a safe drug with relatively limited toxicity which nevertheless has a narrow clinical dose range because of its side effects on the gastrointestinal system. It may be capable of influencing immune function but the changes it induces are relatively small and may not be sufficient to overcome the stronger immunodepressive effects of cancer chemotherapy.

[5] *See* footnote 3.

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Effect of Levamisole as a Surgical Adjuvant Therapy for Malignant Melanoma [1]

Raymond L. Gonzalez, Lynn E. Spitler,* and Richard W. Sagebiel [2,3]

SUMMARY

The preliminary results of a trial in progress are reported. Two hundred patients have been enrolled in a randomized, double-blind, placebo-controlled trial of levamisole in malignant melanoma. A median followup of 20 months has been reached. Endpoints of the study include disease-free interval, interval free of visceral disease, and survival. Overall, there is a trend in favor of the levamisole group with regard to all three endpoints, but the difference is not significant. Significant differences between the control and levamisole groups are found at some, but not all, time intervals when the analysis includes only patients with stage II disease (positive lymph nodes at the initiation of the study). Although these preliminary results are encouraging, at the present time the overall curve for patients with stage II disease is not statistically significantly different when analyzed by the method of Mantel.

[Cancer Treat Rep 62:1703–1707, 1978]

Levamisole has been shown to enhance cellular immune activity in animals (1) and in man (2). Beneficial effects have followed its use in some experimental animal tumors (3–6) but not in others (5). Malignant melanoma in man has clinical and immunologic features which suggest that the course of disease is influenced by the host's immune response. This evidence is reviewed elsewhere (7), and includes the spontaneous regression of either primary or metastatic tumors, the occurrence of metastasis many years after apparent curative treatment of the primary, and the demonstration by a number of methods (delayed hypersensitivity skin testing, lymphocyte blastogenesis, leukocyte migration inhibition, and cell-mediated cytotoxicity) of sensitization to melanoma antigens in early disease and loss of reactivity in progressive disease. The location of the primary tumor on the skin surface allows earlier detection and more adequate resection than is often the case with malignancies arising internally.

These considerations led us to undertake a randomized, double-blind, placebo-controlled trial of levamisole in patients with malignant melanoma. This paper contains the interim results of that study.

MATERIALS AND METHODS

Patients

Two hundred patients were recruited for the study. Some of these individuals had a primary melanoma invasive to levels III, IV, or V (8) with or without a regional lymph node dissection. Other patients had lymph node or skin metastases which appeared > 90 days after excision of a primary melanoma. The remaining patients had either mucosal or subungual primaries or metastases from an

[1] Supported by a gift from Janssen Pharmaceutica and by Public Health Service grant CA-13671 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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[3] We thank Drs. Richard Glogau, Peter Wong, Thomas Malm, Randolph Chase, Robert Allen, Cleo Dymott, Richard Bohannon, Thomas Drake, William Epstein, and Scott Blois for their participation in the care of the patients in this study, and Dr. Byron Brown and Elizabeth Scott for assistance in the statistical analysis.

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TABLE 1.—Categories of patients in a randomized, double-blind trial of levamisole vs placebo in malignant melanoma

Randomization group	Clinical and pathologic criteria
1	Primary melanoma: levels III, IV, V; no node dissection
2	Primary melanoma: levels III, IV, V; negative node dissection
3	Primary melanoma: levels III, IV, V; positive node dissection
4	Recurrent melanoma: local, in transit, or regional lymph nodes > 90 days from primary
5	Recurrent melanoma: remote lymph nodes or skin, completely excised, or unknown primary
6	Mucous membrane melanoma

unknown primary. The patients were divided into six groups (table 1).

All patients were required to be ≥ 18 years of age. All patients had undergone surgical removal of all detectable tumor and had no evidence of malignancy on physical examination, chest roentgenogram, serum enzyme measurements, and brain, liver, and bone or gallium scans. Entry in the trial had to be accomplished within 90 days of the removal of the last detectable tumor. No patient was enrolled who had had prior radiation or chemotherapy or who was receiving immunosuppressive drugs such as steroids. Patients with ocular or level II melanomas were not eligible.

Treatment

At the time of entry in the study, each patient was assigned to one of the six patient groups listed in table 1, and was then randomized within that group to treatment with either levamisole or placebo. This was done in a double-blind fashion. The drug is administered orally at a dose of 150 mg of levamisole (or an indistinguishable placebo tablet) each day for 3 consecutive days every other week. Treatment is continued for 2 years, and if there has been no recurrence by then the agent is stopped. Patients who develop a recurrence continue to take the pills until they have been tumor-free for 2 years or until death. Patients are re-examined at our clinic every 3 months. Suspicious findings may cause more frequent evaluations.

Statistical Analysis

Survival and recurrence rates were calculated after the method of Berkson and Gage (9). Significance testing of differences between these rates in the levamisole and placebo groups was done by the method of Mantel (10). In the analysis of data regarding the distribution of patients, chi-square testing was used. Differences between individual points on the life-table curves were analyzed by the large sample normal approximation for testing the differences between two estimates when the standard errors are known.

Two-tailed distributions were used in all determinations of P values.

RESULTS

Patient distribution.—When the patients receiving levamisole and those receiving placebo were examined with respect to age, sex, randomization group, and the level of invasion of the primary tumor, no important differences were found.

First recurrence.—At the time of this analysis, the median followup was 20 months. There was found to be a small and statistically nonsignificant trend in favor of the levamisole group.

First visceral recurrence.—There was no statistically significant difference between the levamisole and placebo groups when rates of visceral metastasis were compared.

Survival.—Although there did appear to be a trend in favor of the levamisole group, no statistically significant differences were found.

Stage II patients.—The presence of low-risk patients might obscure a therapeutic effect in high-risk patients. All patients with lymph node metastases (stage II) at the time of entry were analyzed separately. There were 47 such patients in the levamisole group and 43 in the placebo group. When individual time intervals were considered, there were several points where statistically significant differences between the levamisole and control groups were present with respect to the appearance of the first recurrence, first visceral recurrence, and survival (figs 1–3). This was most striking in the survival curves (fig 3). All differences were in favor of levamisole. When all curves were analyzed by the method of Mantel (10), the differences were not significant. Overall, at the present time, the differences between the groups is of borderline significance and favors levamisole. In terms of the time to first recurrence, the difference between the groups was more striking early, and became less with additional time.

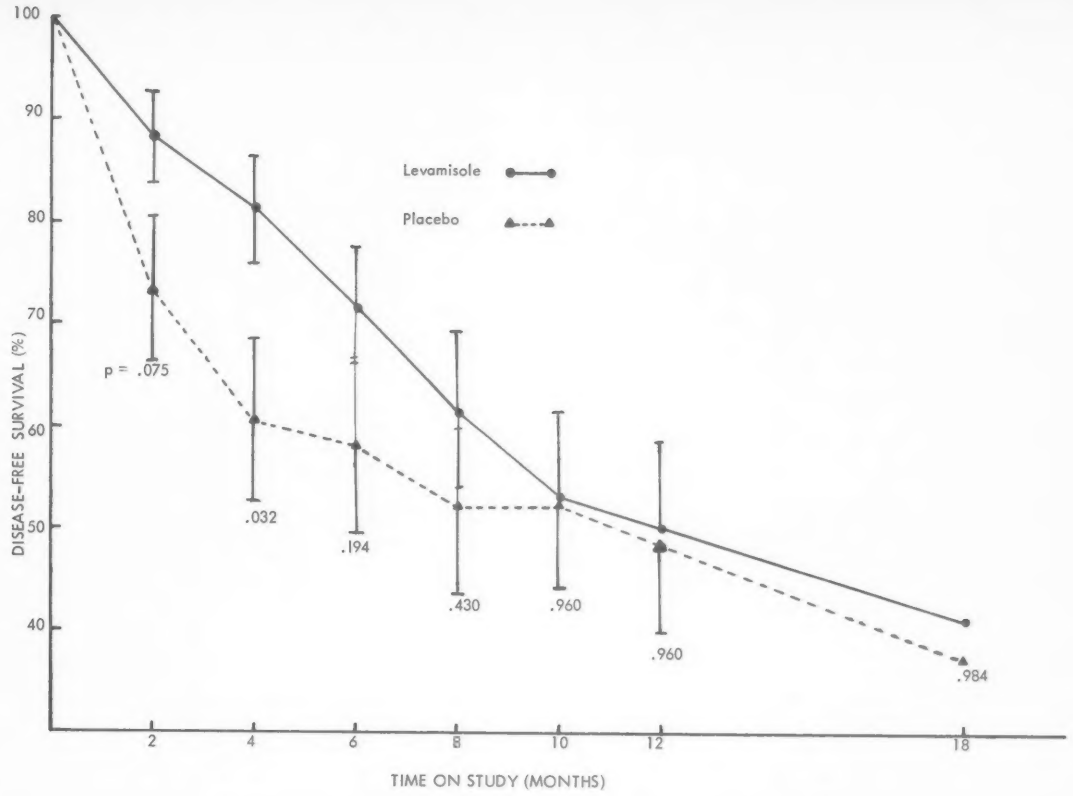

FIGURE 1.—Disease-free probability rate of patients with stage II melanoma randomized to levamisole or placebo. Bars = standard errors; Nos. = 2-tailed *P* values at each point.

DISCUSSION

This report reflects the preliminary results of a randomized trial of levamisole versus placebo in patients with malignant melanoma. No significant difference between the two groups is apparent when all patients entered are analyzed but a definite trend is observed when only patients with stage II disease are analyzed.

Detection of a drug effect might have been made more difficult by the presence of low-risk patients. The patients in randomized group 2 (negative lymph node surgery) have an excellent prognosis whether treated with levamisole or placebo. This prognosis is shared by the approximately 75% of patients in randomization group 1 who would have had negative lymph nodes if surgery had been done. Inclusion of such low-risk patients, probably comprising 30% of the patient sample, would be expected to reduce the statistical impact of differences in the groups with higher risks. This fact is illustrated by the results seen in the stage II patients.

The result observed in the patients with stage II disease (that there was a large difference between the levamisole and control groups early, which became less with additional time) is of interest. This could be due to the development of pharmacologic tolerance, tachyphylaxis, or accelerated drug metabolism.

It must be emphasized that these results are preliminary and that less than half of the patients have completed the 2-year course of medication. The emergence of a trend in favor of the levamisole group at this time is very encouraging. The data are insufficient, however, to justify a pronouncement on the efficacy or lack thereof of adjuvant levamisole therapy in malignant melanoma.

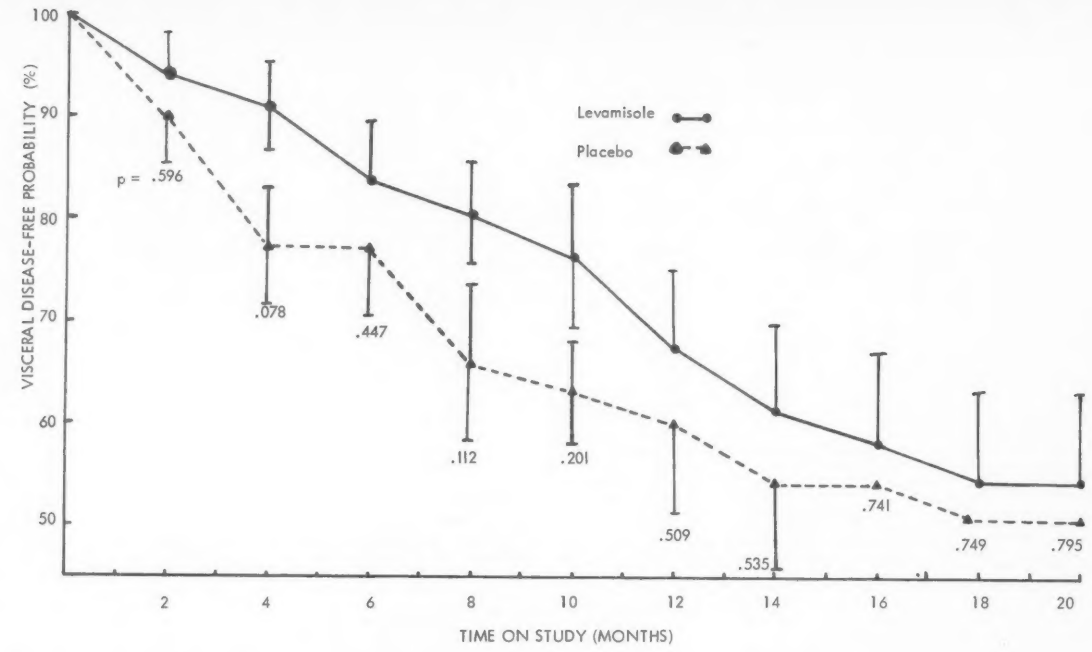

FIGURE 2.—Probability of no visceral recurrence in patients with stage II melanoma randomized to levamisole or placebo. Bars = standard errors; Nos. = 2-tailed *P* values.

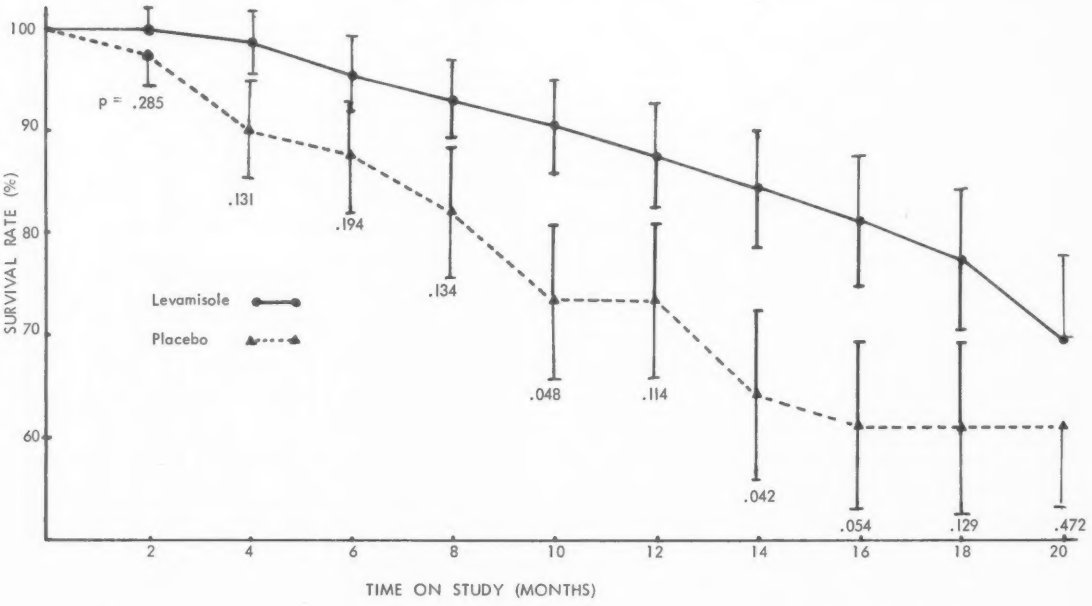

FIGURE 3.—Cumulative survival rate of patients with stage II melanoma randomized to levamisole or placebo. Bars = standard errors; Nos. = 2-tailed *P* values.

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Preliminary Report of the Use of Levamisole in the Treatment of Bladder Cancer

Robert B. Smith,* Jean deKernion, Barbara Lincoln, Donald G. Skinner, and Joseph J. Kaufman [1]

SUMMARY

Sixty-two patients with transitional cell carcinoma have been admitted to a double-blind randomized control study with levamisole as an immune adjuvant, in addition to standard therapy for noninvasive and invasive bladder cancer. Levamisole has been shown to be easily administered and is well-tolerated, especially when compared to other immune adjuvants such as bacillus Calmette-Guerin or *Corynebacterium parvum*. To date, there is no significant difference in the disease-free interval in the levamisole-treated group compared to the placebo group. Initial dinitrochlorobenzene (DNCB) reactivity may be an important prognostic indicator with regard to tumor recurrence. Tumor recurrence seems to be rare in those patients who are initially DNCB-positive. Total monocyte count, T lymphocyte, FC receptor cells, and PHA response showed no improvement with levamisole therapy. Monocyte chemotaxis remains the only immune function study to improve with levamisole, but the clinical significance of this test is yet to be explained.

[Cancer Treat Rep 62:1709–1714, 1978]

Although levamisole (an imidazo-thiazole derivative) has been used for many years as an anthelmintic, it was not until 1971 that Renoux and Renoux (1) found this agent to have possible immunostimulative effects. Many studies were instituted as a result of this observation, especially in the immunodeficient patient (2,3). Clinical studies suggested that levamisole seems to be most effective in patients who have small tumor burdens and who are compromised immunologically by radiation or chemotherapy. Levamisole affects primarily T-cell and phagocyte functions (cell-mediated arm of the immune system) (4,5). The use of levamisole as an adjunct to surgery in resectable lung cancer has shown a significant increase in survival and disease-free interval over control patients (6,7). Rojas and co-workers (8) have found a similar significant prolongation in disease-free interval and survival in patients with stage C breast tumors who were treated with levamisole and extensive radiation therapy.

There has been little information with regard to the benefits of levamisole in carcinoma patients with large tumor burdens, although patients who do not appear to respond to chemotherapy appear to gain little benefit from levamisole.

Patients with transitional cell carcinoma of the bladder who have minimal residual disease are candidates for immunostimulation for several reasons. First, radiation therapy, which is an integral adjuvant therapy for this tumor, causes immunosuppression for a variable period of time. Second, there is a 30% 5-year survival expectancy in such patients (with muscle invasion). This poor survival can be attributed to the presence of widespread micro-foci of tumor at the time of definitive radiation therapy and surgery. Third, there have been no proven effective chemotherapeutic agents for the treatment of advanced bladder cancer, and therefore the prevention of disseminated and local recurrence is highly desirable.

Patients with stage A recurrent malignant bladder papillomata are also ideally suited for adjuvant

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immunotherapy. At least 50% of these patients will develop recurrent tumors, and only 30%–40% will respond to adjuvant thioTEPA therapy. Furthermore, as the number of recurrences increases or if carcinoma in situ is present, the risk of developing an invasive and anaplastic carcinoma markedly increases and between 30% and 50% of such patients can be expected to eventually require radical surgery and radiation therapy. Levamisole appears to be an appropriate agent for the treatment of these two types of bladder tumors, since the drug is relatively innocuous and therefore can be justified as adjuvant therapy, even in patients with noninvasive cancer. The high incidence of recurrence in both of these stages of bladder cancer further justifies the need for adjuvant therapy. This study is therefore designed to evaluate whether levamisole will reduce the rate of recurrence of invasive bladder cancer following definitive surgery and radiation therapy and whether it will decrease the recurrence rate and lengthen the disease-free interval in patients with recurrent noninvasive bladder cancer.

MATERIALS AND METHODS

Patients with transitional cell carcinoma of the bladder were admitted to the study and subdivided into the following groups: (*a*) patients with noninvasive tumors, and (*b*) patients with invasive bladder tumors treated either with a partial or total cystectomy, who were felt to have minimal residual disease. In addition to our usual therapy for these patients, each group was individually randomized in a blind fashion to receive either levamisole or an identical placebo. Patients with diffuse carcinoma in situ were also admitted to the study as a separate group and were given levamisole. Patients with diffuse carcinoma in situ who were treated with radical surgery were placed in a separate group from those with the bladder left intact. Since the natural history of carcinoma in situ left in place is so predictable and since no effective chemotherapeutic or radiation therapy program has been successfully devised, we felt a placebo group was not necessary in this population. No information exists, however, as to whether these patients represent good candidates for adjuvant immunotherapy.

Patients who had a second malignancy or a life expectancy of < 3 years and patients > 80 years of age irrespective of their bladder tumor were excluded from this study. In addition, patients with known metastatic disease or known residual local tumor after definitive therapy were not considered candidates for the study.

TABLE 1.—Time at risk of the patients in each tumor category

Time (mos)	Total No. of patients	No. of patients with carcinoma stage—		
		Superficial	Invasive	In situ
1	62	31	23	8
3	49	23	18	8
6	34	16	12	6
9	24	11	10	3
> 12	16	7	7	2

Patients received 2.5 mg/kg of either placebo or levamisole each day for 2 consecutive days each week (rounded off to the highest 50-mg increment). We attempted to start this medication prior to surgery or radiation therapy but were not successful in all instances. Treatment continued on a blind basis for a minimum of 1 year, or a maximum of 2 years, or until tumor recurrence, metastasis, or drug toxicity was noted. The randomization code was broken at that time. Appropriate laboratory studies, including cbc, platelet and absolute lymphocyte counts, chest roentgenogram, creatinine, and SMA-12 were performed every 3 months. Liver and bone scans were performed yearly, and more often if clinically indicated. Iv pyelograms were performed at 6-month intervals for 2 years and yearly thereafter. Skin tests for delayed cutaneous hypersensitivity and in vitro immune function tests were repeated every 8 weeks.

RESULTS

This is a preliminary report. A total of 62 patients have been admitted to this study in the past 2 years. Followup ranges from 1 month to 2 years. Patient entry continues to be active. Table 1 illustrates the time at risk of the patients in each tumor category. Thirty-one patients with noninvasive transitional cell carcinoma have been admitted to the study. Twenty-three patients with invasive cancer have been admitted; in addition, eight patients with carcinoma in situ are being studied. Four patients with in situ carcinoma and a history of prior lesions have had radical cystectomies and have been placed in the "minimal residual disease cystectomy" category for followup.

As expected, there is a strong male preponderance (5 to 1) which was evenly distributed over all tumor stages. Thirty-four patients continue on the protocol; 17 have been removed from the study because of recurrent disease. Four patients were removed from the study because of side effects, and seven patients deviated from the protocol.

Side Effects

Four patients experienced side effects of sufficient severity to require removal from the study. One patient experienced severe neutropenia 4 ½ months after levamisole therapy (200 mg/day for 2 consecutive days each week). A drug rash was noted 2 months prior to the neutropenia episode, and at that time the dosage level of levamisole was halved to 100 mg/day for 2 days each week. No diminution in the rash was noted. The drug was discontinued 4½ months after the initiation of therapy when the wbc count was noted to be 861/mm^3 with 3% segmented neutrophils and 1% bands. Forty-eight hours later the wbc count reached its nadir at 477/mm^3 (3% segmented neutrophils). The patient was isolated, and over the subsequent 3 weeks the wbc count increased to 1000/mm^3; 5 days later the wbc count increased to 3900/mm^3 when the patient was discharged. One week following discharge, the patient's wbc count normalized (8700/mm^3), with a normal differential. An antineutrophil antibody has been identified in this patient, which was cytotoxic to his neutrophils as well as to neutrophils of random donors. Another patient noted a modest decrease in the wbc count to 3600/mm^3, also associated with a pruritic rash and a positive antineutrophil antibody. Levamisole was discontinued at the patient's request. (This patient had previously been converted to levamisole when he had severe side effects from placebo.) Antineutrophil antibody has been identified in other study patients, but they have yet to develop any evidence of neutropenia.

The other two patients who had to be removed from the study experienced flu-like syndromes 2 weeks after the initiation of therapy, consisting primarily of lethargy, nausea, vomiting, headache, and fever. The levamisole was withdrawn and the symptoms rapidly disappeared. They have not been re-challenged with levamisole.

In addition, mild transient side effects have been noted (table 2) in other patients, but were not severe enough to warrant cessation of therapy. These minimal side effects include flu-like syndrome, insomnia, lethargy, anorexia, metallic taste, and euphoria. The importance of including a placebo in a study such as this is demonstrated by the fact that seven patients receiving placebo experienced side effects. One of these patients had side effects severe enough to require removal from the study.

TABLE 2.—Side effects

Drug	Side effect	No. of patients
Levamisole	Severe	
	Flu-like syndrome	1*
	Leukopenia	1*
	Moderate	
	Flu-like syndrome	1*
	Insomnia	1
	Leukopenia	1
	Skin rash	5
	Lethargy	3
	Mild	
	Metallic taste	5
	Lethargy	5
	Euphoria	3
	Insomnia	4
	Anorexia	2
	Diarrhea	1
	Totals	
	None	13
	Mild	14
	Moderate	6
	Severe	2*
Placebo	None	16
	Mild	4
	Moderate	2
	Severe	1*

* Removed from study (3 patients).

Tumor Recurrence

Thus far, in our study group we have been unable to confirm any beneficial effects of levamisole over placebo with regard to recurrence rate. Of the superficial tumor patients, seven of 17 levamisole patients have had recurrence, whereas six of 13 placebo patients have had recurrence. There was no artificial bias in this study regarding tumor histologic grade (table 3) within each treatment subgroup. The mean time of recurrence in the levamisole group was longer (173.6 versus 132.5 days), but this was not significant. Three patients who had recurrence while receiving placebo have been converted to levamisole. It is of interest that these patients had rapid recurrence while receiving placebo (75, 90, and 120 days respectively) and are now receiving levamisole 90, 180, and 360 days later without any subsequent recurrence.

Similar results are seen in the minimal residual disease invasive group after cystectomy, with four of 16 levamisole patients having recurrence and two of 12 placebo patients having recurrence, again with no significant difference in disease-free interval. Figure 1 shows the disease-free interval for all stages, which is representative for the individual groups of superficial and invasive lesions. It is interesting that only one patient with carcinoma in situ among those patients with the bladder left intact has had recurrence, measured either by bladder biopsy or urine cytology.

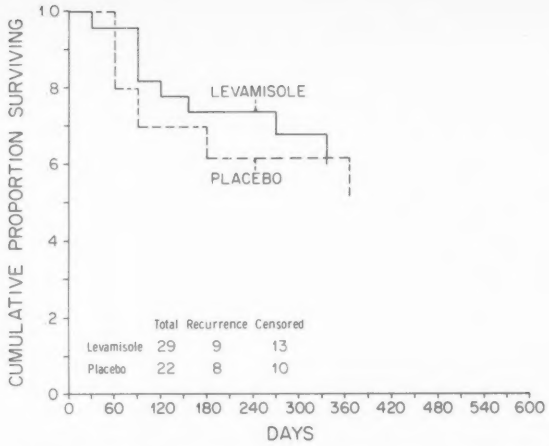

FIGURE 1.—Disease-free interval for all tumor stages, comparing levamisole-treated group to placebo group. No significant difference was noted between the 2 groups. No significant difference in disease-free interval was noted when superficial and invasive tumors were considered as separate groups.

Immunologic Data

Dinitrochlorobenzene (DNCB) reactivity was investigated. In patients with followup long enough to evaluate, an initial positive response was seen in seven of 23 patients with superficial tumors (30.4%) and in only three of 24 patients with invasive carcinoma (12.5%). There also seemed to be a definite trend that patients with an initially negative DNCB skin test had a higher rate of recurrence, whether they were treated with levamisole or placebo. The recurrence rate in those patients who were initially DNCB-positive was 10% (one of 10), compared to 37.8% (14 of 37) in those patients who were initially DNCB-negative. This trend was evident in both the superficial and invasive groups. The treatment category did not seem to influence this. It is interesting that no levamisole patient with an initial positive DNCB test had a tumor recurrence; however, the numbers are much too small to make any conclusion from this (figs 2–4). In addition, those patients who had an increase of ≥ 3 in the level of DNCB response seemed to have a lower recurrence rate than those with a ≤ 1 increase in the level of response (table 4). Many patients in both the levamisole and placebo groups noted an increase in DNCB reactivity after therapy, which most likely was related to a decrease in tumor burden rather than to any specific effect of the treatment agents.

The correlation of absolute lymphocyte count has been disappointing in relation to stage and grade of the tumor and response to therapy. No definite pattern can be found with regard to increase in absolute lymphocyte count and response to levamisole therapy or in relation to the patient with recurrent disease. In addition, total monocyte count, T lymphocyte, FC receptor cells, and phytohemagglutinin response have all been monitored, and there have been no increases in these immune function studies in the levamisole patients compared to the placebo group (9).

The only immune parameter with any positive correlation with levamisole administration was

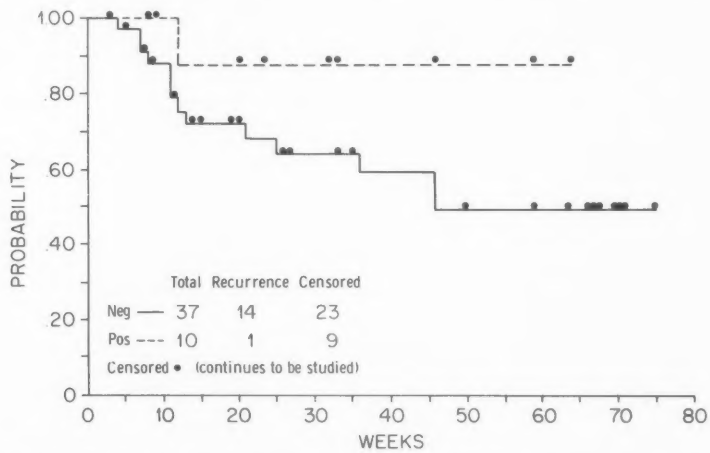

FIGURE 2.—Disease-free interval related to initial DNCB response for entire series including all tumor stages. DNCB-positive is > 4 mm erythema or induration with 25 μg of DNCB.

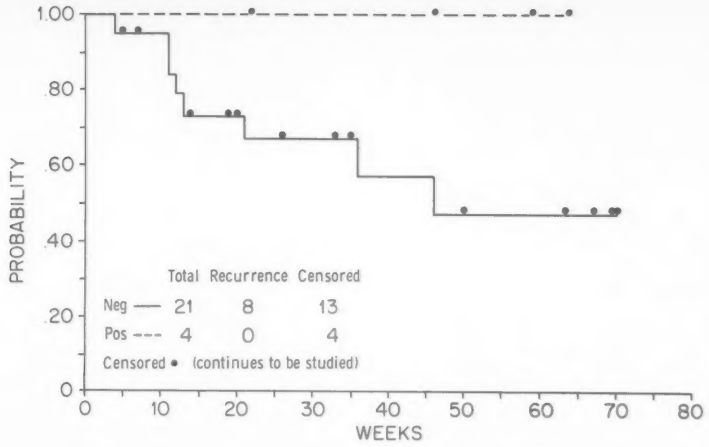

FIGURE 3.—Disease-free interval related to initial DNCB response for levamisole-treated patients, all tumor stages.

monocyte chemotaxis. This was performed in these patients by Nathanson at the University of California at Los Angeles.[2] Fourteen study patients were examined in a double-blind fashion. All ten levamisole patients tested had an increase in chemotaxis, whereas none of the four placebo controls or five non-age-matched controls had increased chemotaxis. As yet we have been unable to explain the significance of this finding. Monocyte chemotaxis, at least in this study, appears to have no relevance with regard to clinical course of bladder tumor patients.

[2]Nathanson D. Personal communication, 1977.

DISCUSSION

The number of patients and length of followup are obviously too small to permit any statements with regard to the efficacy of levamisole as an immune adjuvant in the treatment of bladder cancer. In this study, we have shown the drug to be easily administered and well-tolerated with few side effects, especially when compared to other methods of immunotherapy, such as bacillus Calmette-Guérin and *Corynebacterium parvum*. Patient acceptance is vastly superior compared to other immunotherapeutic agents with which we have had experience. Nevertheless, these early results are

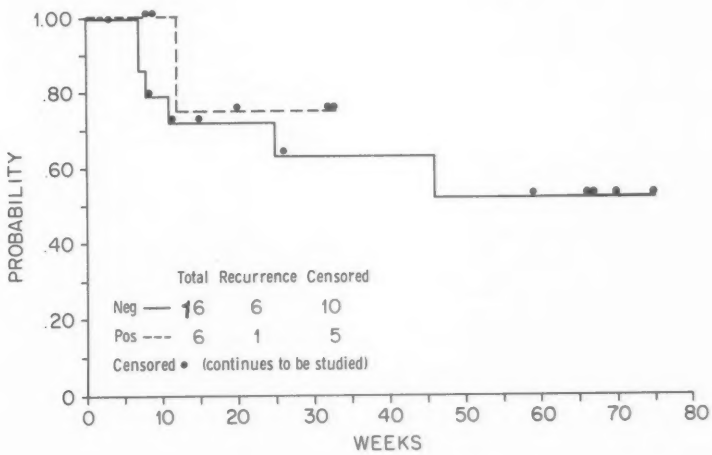

FIGURE 4.—Disease-free interval related to initial DNCB response for placebo-treated patients, all tumor stages.

TABLE 3.—Histologic grade related to recurrence in superficial tumor patients

Category	No. of patients with histologic grade—			
	1	2	3	4
Levamisole (continued treatment)	3	5	2	0
Placebo (continued treatment)	4	2	0	1
Levamisole (recurrence)	1	4	1	0
Placebo (recurrence)	2	2	1	0

TABLE 4.—Recurrence rate related to increase in DNCB reactivity

	No. of patients with an increase of ≥ 3 in level of response			No. of patients with an increase of ≤ 1 in level of response	
	Continued treatment	Recurrence (%)		Continued treatment	Recurrence (%)
Total patients					
Levamisole	7	3 (30%)	Levamisole	2	8 (80%)
Placebo	6	4 (40%)	Placebo	4	5 (55.5%)
Superficial tumor					
Levamisole	4	1 (20%)	Levamisole	1	6 (85.7%)
Placebo	3	4 (57.2%)	Placebo	2	2 (50%)
Invasive tumor					
Levamisole	3	2 (40%)	Levamisole	1	2 (67%)
Placebo	3	0 (—)	Placebo	2	3 (60%)

somewhat disappointing because there is not a significant increase in the disease-free interval in the levamisole-treated patients.

The trend toward a delay in the recurrence time in the superficial tumor patients is interesting and correlates somewhat with the work of Lamm et al[3] in which levamisole demonstrated the ability to significantly delay the onset of transplanted transitional cell carcinoma in C3H mice. It may be that the optimum dose of levamisole to cause immunostimulation in these patients has not yet been defined.

It was also disappointing to see that immune function tests such as phytohemagglutinin response, FC receptor cells, and T lymphocytes were not improved with levamisole therapy. The finding of an increase in monocyte chemotaxis in the levamisole-treated patients remains interesting. Hausman and Brosman (10) demonstrated defective monocyte function in patients with bladder cancer, but were unable to find a correlation between stage of tumor and degree of chemotactic defect. Similarly, in our study, the increase in monocyte chemotaxis did not seem to favorably influence the outcome with regard to tumor recurrence.

[3]Lamm DL, Yee GN, Reichert DF, et al. Levamisole immunotherapy of experimental transitional cell carcinoma. Submitted to *Urology*, 1978.

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Correlations of Clinical and Laboratory Effects of Treatment With Levamisole in Autoimmune Disease [1]

Joshua Levy [2,3,*]

SUMMARY

Studies in which levamisole has been used with success in the treatment of rheumatoid arthritis and other autoimmune diseases may provide information useful in cancer treatment with regard to optimal dosage regimens and methods for monitoring laboratory effects. Using the response of peripheral blood lymphocytes to phytohemagglutinin mitogen measured by the uptake of tritiated thymidine in 3-day culture, patients with rheumatoid arthritis were found to have a significantly deficient response compared to normal subjects. Levamisole treatment reversed this deficiency and normalized lymphocyte mitogen responses. These studies showed the benefit of a dose of 150 mg of levamisole compared to lower doses and also showed equal maintenance of immunocompetency with a dose of 150 mg/week compared to daily levamisole. In addition, compared to poor clinical responders, good clinical responders were shown to have a greater enhancement in mitogen response, both during the treatment period and when levamisole was added in vitro to lymphocyte culture prior to drug treatment. It was concluded that laboratory studies of lymphocyte mitogen response may contribute towards better management of patients receiving levamisole therapy.

[Cancer Treat Rep 62:1715–1719, 1978]

Despite the possible differences in immunologic abnormalities between neoplastic and autoimmune disease, the use of the immunomodulator, levamisole, brings forth a number of pertinent questions which may be appropriate to both conditions. These questions include:

1. What is the optimal dosage regimen for levamisole treatment?
2. Is there an assay which may be used to monitor the effects of levamisole and to assist clinicians in optimizing the treatment program for an individual patient?
3. Is there any in vitro assay that might be helpful in screening patients prior to treatment to predict the likelihood of a good response?

Answers to these questions gained from experience in using levamisole in autoimmune disease might have application towards similar questions relevant to cancer immunotherapy.

Despite our inability as yet to characterize the etiology of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, an impressive accumulation of data points to immunologic mediation of the disease process. Increased IgG synthesis (1), the findings of immune complexes in synovial fluid and serum and deposits of these immune complexes in injured tissues such as the real glomerulus (2), the finding that such immune complexes could fix complement, and evidence for reduced serum complement in active systemic lupus erythematosus (3) all point to an overactivity of humoral immunity in these patients and to a possible loss of normal tolerance and immunoregulation.

Recent work in animal models, particularly the NZB mouse model of human lupus, has suggested that tolerance to self is maintained actively as a function of a population of lymphocytes known as suppressor T cells (4). Deficient suppressor T-cell

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[3]The technical assistance of Kyoung Lee and the clerical assistance of Evelyn Tackels are gratefully acknowledged.

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function in animals has been associated with enhanced B-cell function, a loss of self tolerance, and the appearance of circulating autoantibodies (5,6). The association of B-cell overactivity and autoantibodies with a T-cell defect may also apply to the human autoimmune diseases of rheumatoid arthritis and systemic lupus erythematosus. Indeed, T-cell deficiencies have been described in both rheumatoid arthritis and systemic lupus erythematosus (7,8).

Levamisole, a drug originally designed as an anthelmintic agent which is currently being investigated in a wide variety of disorders, has been shown in a number of carefully controlled studies to have significant therapeutic benefit in rheumatoid arthritis (9–11). A suggested mechanism of action for the drug in rheumatoid arthritis (in which T-cell deficiencies have been described) is stimulation of cell-mediated immunity with presumed enhancement of suppressor T-cell function controlling autoimmune phenomena and immunoglobulin synthesis. Studies reported from our laboratory and by others have shown levamisole to stimulate cell-mediated immunity (12–17). These studies in both autoimmune and neoplastic diseases show similar deficiencies of T-cell function and frequent reversal of these deficiencies with levamisole. In our own laboratory we have found deficient T-lymphocyte responses to mitogens, particularly suboptimal doses of phytohemagglutinin (PHA) in rheumatoid arthritis, and found these deficiencies to be reversed with both in vitro and in vivo levamisole.

Peripheral blood lymphocytes were separated by Hypaque-Ficoll separation and were adjusted to a mononuclear cell concentration of 1×10^6 cells/ml of medium containing RPMI-1640, Hepes, and 10% fetal calf serum. Two and one-half million cells were cultured in 2.5 ml with serial dilutions of 0.025 ml of Difco PHA-M per ml. After 72 hours the cells were washed, resuspended in 1.5 ml of medium without fetal calf serum, and labeled with 0.5 microcurie of tritiated thymidine for 3 hours at 37°C. The cells were then treated with 10% trichloroacetic acid, dried, dissolved in 0.3 ml of 5 N sodium hydroxide, and decolored with hydrogen peroxide. One hundred lambda of the mixture was added to 15 ml of liquid scintillation fluid and was counted for 1 minute in a Nuclear Chicago liquid scintillation counter. Normal subjects responded to an optimal dose of PHA with approximately 3000 cpm/500,000 cells and a normal unstimulated culture produced only 150 cpm. The mean value for 22 normal subjects was found to be 2700 cpm. In 13 patients with rheumatoid arthritis, the mean lymphocyte response was 20% below normal at the optimal dose of PHA, for a P value of < 0.02, and 46% below normal at the suboptimal dose of 1:32 dilution of PHA, for a P value of < 0.001.

Following these initial observations, we then applied the same methodology to another group of patients with rheumatoid arthritis participating in a double-blind controlled study of levamisole versus placebo. The protocol called for patients to be randomized to start on either drug or placebo and to be crossed over at 16 weeks of therapy. The dose of levamisole or matched placebo administered was 150 mg/day. Concomitant drug therapy which was permitted included analgesics and anti-inflammatory drugs, but all drugs were maintained at a constant level throughout the study. Twenty patients were entered in the study who fulfilled the American Rheumatism Association criteria for classic or definite rheumatoid arthritis. No patients were entered who were receiving immunosuppressives, antimalarials, gold, or penicillamine. In vitro culture of patients' lymphocytes with PHA was performed serially during the drug trial.

Prior to drug treatment, this group of patients showed a significantly deficient mean response to PHA. The effect of levamisole over the first 8 weeks of treatment is summarized in figure 1. Levamisole (150 mg/day orally) induced a rapid highly significant increase in PHA response as early as 1 week following initiation of therapy. P values showing the significance of the difference between drug and placebo by Student's t test analysis are shown in the figure. Patients treated with placebo continued

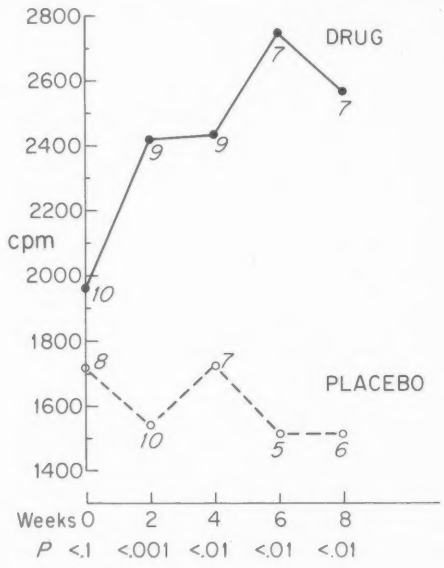

FIGURE 1.—Lymphocyte PHA mitogen responses over the first 8 wks of treatment with both levamisole and placebo. The mean normal response in 22 controls is 2700 cpm/0.5-million lymphocytes.

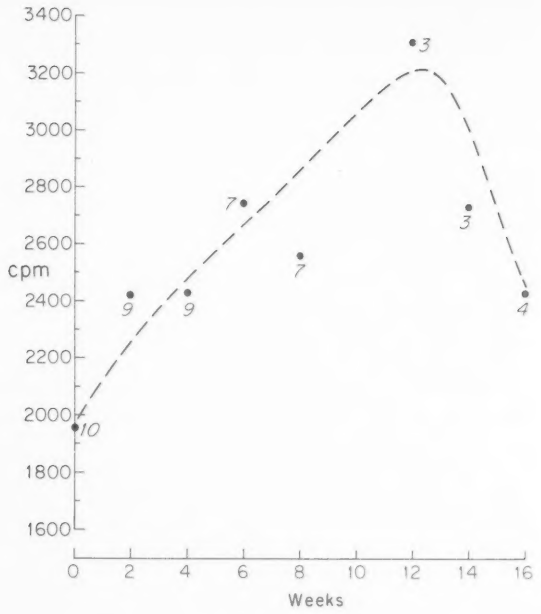

FIGURE 2.—Lymphocyte PHA mitogen responses in patients receiving levamisole over 16 wks of treatment.

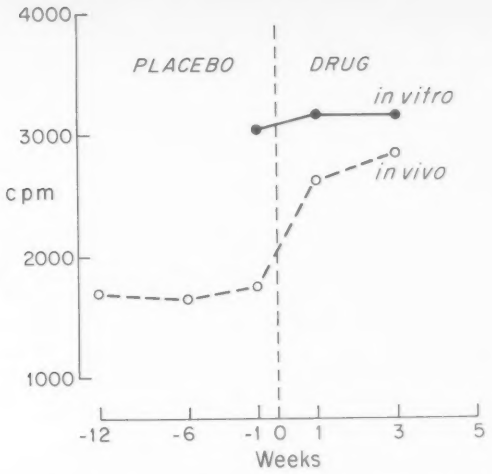

FIGURE 3.—Lymphocyte PHA mitogen responses before and after levamisole treatment. The upper curve = the patient's lymphocytes cultured with added in vitro levamisole (1 μg/ml of culture medium).

to show a deficient mean response with little change from their initial values. Most patients receiving levamisole, however, reached a normal mitogen response and maintained it for the first 8 weeks of observation.

Although the number of patients receiving levamisole for > 8 weeks was small, a trend towards a decrease to original baseline values was seen during the last 8 weeks of treatment (fig 2). Possible explanations for this phenomenon are that either patients acclimated to the drug or that the immunostimulant exhausted the T lymphocytes over this long period of daily treatment. These hypotheses could have been tested by increasing the oral dosage of levamisole and observing the lymphocyte response. We were, however, limited by protocol from increasing the dose of levamisole in this study in rheumatoid arthritis.

We had an opportunity to explore this question in another group of patients. In collaboration with the University of California at Los Angeles Multiple Sclerosis Study Group, we investigated lymphocyte mitogen response in patients with multiple sclerosis and observed a deficiency similar to that in rheumatoid arthritis. During an open trial of levamisole in multiple sclerosis we were able to serially follow patients being treated with levamisole in an increasing stepwise dose regimen. Although patients showed an initial deficient response to PHA, an improvement in mitogen response was observed with 50 mg of levamisole; in subsequent weeks, however, this effect was lost. Levamisole was then increased to 100 mg/day and lymphocyte response was again greatly enhanced. When mitogen response again began to fall, the patient's dose was increased to 150 mg and the patient again showed a return to normal levels of PHA lymphocyte stimulation. This course of lymphocyte response in relation to drug dosage would appear to speak against immune exhaustion as an explanation for the late decreases in lymphocyte response to this drug, and may suggest that acclimation or tachyphylaxis would better explain the decreasing lymphocyte response. Serial followup with laboratory assessment of T-cell function in patients being treated with levamisole might also permit appropriate drug adjustment to maintain patients in the range of normal T-lymphocyte response. An important question to be asked is, does the improvement in lymphocyte response correlate with clinical effect in rheumatoid arthritis? Overall, the results of trial showed statistically significant improvement in parameters of disease activity such as active joint count, grip strength, and duration of morning stiffness in levamisole-treated patients compared to placebo, and a significant decrease in sedimentation rate and latex fixation titer for patients treated with drug. Improvement, however, was not uniform and

TABLE 1.—% enhancement of mitogen response before vs after levamisole treatment (mean ± SE)

	PHA 1/16	PHA 1/32
Clinical responders (9 patients)	36.9 ± 4.7	34.5 ± 5.5
Clinical nonresponders (8 patients)	32.2 ± 3.9	23.2 ± 7.8 ($P < 0.05$)

TABLE 2.—% enhancement with in vitro levamisole prior to beginning drug therapy (mean ± SE)

	PHA 1/4	PHA 1/32
Clinical responders (9 patients)	62.8 ± 5.6	44.8 ± 4.8
Clinical nonresponders (8 patients)	51.0 ± 5.8	32.7 ± 2.9 ($P < 0.05$)

patients could be divided into good clinical responders (nine) and poor clinical responders (eight). T-cell function was compared in the two groups of differing clinical response. Table 1 shows that patients who have marked clinical improvement had a significantly greater increase in T-cell response during drug therapy than those who did not show clinical improvement.

Our laboratory also had the opportunity to observe the effect of levamisole added in the culture tube to lymphocytes from both normal subjects and rheumatoid arthritis patients before and after receiving oral drug. We found the optimal levamisole in vitro concentrations to be 1 μg/ml of culture medium. While subjects with a normal PHA mitogen response show only a minimal increase in response when levamisole was added to the culture tube, patients with deficient responses show significant improvement often into the normal range.

This effect is demonstrated in figure 3. This patient with rheumatoid arthritis was studied serially, first receiving placebo and then receiving levamisole. Lymphocytes in each time point were cultured with and without the addition of added levamisole. The upper curve represents the lymphocyte response observed following the in vitro addition of drug. We noted that the addition of in vitro levamisole to the lymphocyte culture produced a marked enhancement of mitogen response during the period prior to oral drug therapy, while the patient's lymphocytes were showing a deficient innate response. Following the initiation of oral levamisole, this patient's lymphocytes innately showed a marked improvement in mitogen response and showed only a minimal further increase with added in vitro drug.

Does the extent of a patient's response in vitro to levamisole predict a satisfactory laboratory improvement in clinical response with oral drug? Analysis of our findings in the 17 patients who concluded the double-blind control study of rheumatoid arthritis with levamisole showed that greater lymphocyte enhancement by in vitro levamisole testing prior to starting drug was observed in patients who subsequently had good clinical improvement compared to those patients who showed poor clinical response (table 2). These results encourage us to believe that it may be possible to select patients for immunoregulatory drugs like levamisole by first assessing with in vitro studies the drug's effect on the patients' lymphocytes.

With regard to determining recommendations for dose regimens for the use of levamisole in general, these studies and others of T-cell function with therapeutic programs in our laboratory have suggested that a single dose of 150 mg of levamisole may reverse T-cell deficiency for as long as 3 weeks. A single dose of 150 mg was also shown to have a higher and longer sustained effect than either 50 or 100 mg. An intermittent-dose regimen of 150 mg/week or every 2 weeks in a number of patients currently under study has shown maintenance of normal lymphocyte response for $\geq$ 6 months without any evidence of tachyphylaxis. Because of the decreased likelihood of toxicity and the apparent effectiveness of this intermittent regimen, we currently recommend a dose of 150 mg/week or every 2 weeks for the treatment of autoimmune disease.

In conclusion, we have found that a number of questions regarding the use of levamisole in autoimmune disease may be answered by studying T-lymphocyte function with and without in vitro drug prior to starting a treatment program. Patients more likely to respond clinically may be selected and treatment may be optimized by using laboratory lymphocyte culture techniques. These laboratory studies have contributed towards our current recommendation of 150 mg/week or every 2 weeks for treatment with levamisole. A similar approach may be as successful in the area of tumor immunotherapy.

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Adverse Reactions to Levamisole

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SUMMARY

The adverse reactions mentioned in 267 reports on levamisole are analyzed. Significant adverse reactions are agranulocytosis, skin rash, and febrile illness. They occur in a small subpopulation of patients only, predominantly those with rheumatoid arthritis. Other side effects rarely necessitate discontinuation of treatment. Levamisole did not appear to cause renal or liver toxicity. By means of a questionnaire, cases of blood dyscrasia were analyzed in detail. Agranulocytosis (< 20% neutrophils) occurs most frequently in patients with rheumatic diseases, in women, and in HLA B27 genotypes. It is spontaneously reversible when treatment is discontinued. The bone marrow is not damaged. Thrombocytopenia has a course similar to that of agranulocytosis but it is very rare. Leukopenia (< 3000 cells/mm^3 but > 20% neutrophils) seems to differ from agranulocytosis in a number of ways and is not an indication to discontinue treatment. It is concluded that, if used under the close supervision of a physician, for those clinical situations in which levamisole has proven efficacy, the potential benefit to the patient outweighs the hazards and justifies its use.

[Cancer Treat Rep 62:1721–1730, 1978]

Levamisole has been used extensively as an anthelmintic in man. Controlled studies have shown that the therapeutic dose, given in a single intake, is devoid of side effects (1). Early reports on levamisole used to treat cancer or recurrent infections seemed to confirm the safety of the drug, but treatment periods were relatively short.

The first reports on serious side effects concerned patients with rheumatoid arthritis who had been treated daily for several months (2–4). Published case reports concerning levamisole-induced adverse reactions tend to magnify the significance of side effects if not projected against the total population treated. Therefore, we have carefully analyzed the adverse reactions in the nonanthelmintic literature on levamisole. In addition, we have designed a detailed questionnaire which was sent to each investigator whose patients had experienced agranulocytosis or leukopenia. The results of these investigators are discussed in this paper.

ADVERSE REACTIONS TO LEVAMISOLE

We retrieved 267 published and unpublished reports on 6217 patients treated with levamisole for various diseases. The presence or absence of side effects is mentioned for 3900 of these patients (table 1). The incidence of all side effects is listed, irrespective of the duration of treatment or the dose and treatment schedules used. Eighty percent of the patients whose side effects were reported were treated for > 3 months.

All adverse reactions to levamisole mentioned in the literature are listed in table 2. Those which occur in at least 1% of the patients are shown in table 3. They can be classified roughly into three groups: idiosyncratic or allergic, neurologic, and gastrointestinal.

Idiosyncratic or allergic adverse reactions are

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[4]We wish to thank Madeleine Debbaudt, Christian Hörich, and Henk Scheijgrond for their considerable help in analyzing the data; Jef Dom and Jean Dony for statistical analysis; Dr. Willem Amery for suggesting HLA testing; and the many investigators who kindly completed the questionnaires.

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TABLE 1.—Review of the literature (Mar 1978)

Disease	No. of reports	No. of patients treated	No. of patients evaluated for side effects
Rheumatic	71	1131	989
Other inflammatory	37	1058	601
Infectious	73	1284	888
Cancer	58	2052	1179
Other	28	692	243
Totals	267	6217	3900

significantly more frequent in patients with rheumatic disorders than in patients with other diseases. Gastrointestinal side effects predominate in cancer patients. Febrile illness, neurologic side effects, and gastrointestinal intolerance occur in all patients, irrespective of the disease. Some side effects seem to be entirely drug-related, while others are also disease-related.

Less than 5% of the patients discontinued treatment for adverse reactions. There is an 18% dropout rate in patients with rheumatic diseases. The major reasons for withdrawal are rash, leukopenia or agranulocytosis, and febrile illness. The withdrawal rate for rash and agranulocytosis, is highest in patients with rheumatic diseases. The dropout rate for febrile illness and gastrointestinal intolerance is comparable in all groups.

Placebo-controlled studies have shown that rash, agranulocytosis, and febrile illness are definitely levamisole-related. Neurologic excitement is slightly more frequent with levamisole than with placebo but rarely necessitates discontinuation of treatment. Gastrointestinal intolerance is as frequent with placebo as with levamisole, even in cancer where the observed incidence is the highest.

TABLE 2.—Qualitative survey of adverse reactions to levamisole (Mar 1978)

Hematologic	*Neurologic*
Agranulocytosis/ leukopenia	Sensory stimulation (hypersomnia, dysgeusia, etc)
Thrombocytopenia	Hyper-alert state
Agammaglobulinemia	Insomnia
Skin and mucosae	Psychic confusion
Rash	Tremor
Vasculitis	*Gastrointestinal*
Mouth ulcers	Nausea
Erythema nodosum leprosum	Gastric intolerance
Lichen planus	Anorexia
Flu-like illness	Vomiting
Febrile illness	Diarrhea
Shivering, chills	Constipation
Sore throat	*Various other*
Myalgia, arthralgia	Proteinuria
Excessive perspiration	Hyperazotemia
Dizziness	Allergic shock
Malaise	Increased transaminases
Headache	Ocular symptoms, iridocyclitis
Apathy	Skin ulceration
Fatigue	Polyuria

AGRANULOCYTOSIS, LEUKOPENIA, AND THROMBOCYTOPENIA

The first cases of agranulocytosis during treatment with levamisole were reported in 1976 (2). Soon thereafter, two deaths were reported in children (3). In April 1977, a group of rheumatologists participating in a multicenter study with levamisole in rheumatoid arthritis estimated that approximately 3% of patients with rheumatic diseases may develop this complication (4).

By means of a questionnaire, data were collected on 88 patients with agranulocytosis (< 20% neutrophils), 43 patients with leukopenia (< 3000 cells/mm^3 but > 20% neutrophils), and three patients with thrombocytopenia (< 50,000 platelets/mm^3). These cases include most of the patients reported in the literature and new cases from ongoing studies.

Agranulocytosis

Most of the cases of agranulocytosis were reported from Europe, where levamisole has been most widely used. Nearly 80% of the patients were women. There was no particular age preponderance. There was, however, a significant genetic predisposition. Table 4 lists the HLA A and B antigens in a European control population, in 28 patients (mainly European) with agranulocytosis, and in six patients with leukopenia or thrombocytopenia. The distribution of all antigens except B27 in the patients with agranulocytosis is similar to the distribution in the general population. B27 occurs in 8% of Europeans and is less frequent in other ethnic groups (5). Its frequency is higher in patients with ankylosing spondylitis, Reiter's disease, juvenile rheumatoid

TABLE 3.—Quantitative survey of adverse reactions to levamisole and withdrawal rate because of side effects (Mar 1978)*

	Cancer (1179 patients)		Infectious (888 patients)		Inflammatory (601 patients)		Rheumatic (989 patients)		Various other (243 patients)	
Adverse reaction	Reaction	Withdrawn	Reaction	Withdrawn	Reaction	Withdrawn	Reaction	Withdrawn	Reaction	Withdrawn
Idiosyncratic										
Skin rash	2.6†	0.9†	1.5†	0.3†	1.7†	0.2†	[14.8]	[7.0]	2.1†	0.4‡
Leukopenia/agranulocytosis	2.0‡	1.4†	0.2†	0.3†	1.0†	0.3†	[4.9]	[4.5]	0.8§	0§
Febrile illness	[4.8]	1.4	1.9§	0.5	2.8	1.5	3.9	1.5	0.4§	0
Mouth ulcers	0.3‡	0	0.1‡	0	0.0§	0	[1.9]	0.3	0.0	0
Neurologic										
Sensory stimulation	3.0‡	0.1	6.1	0	4.8	0	[6.2]	0.5	0.4‡	0
Hyper-alert state	1.2	0	1.2	0	0.2§	0	[1.8]	0	0.0	0
Insomnia	[2.5]	0.1	0.3‡	0	0.3§	0	1.4	0	0.0	0
Headache	2.1	0	2.3	0	2.2	0	1.3	0.2	0.0	0
Dizziness	1.2 ‡	0	0.5†	0	0.3†	0	2.9	0	[4.5]	0
Fatigue	[4.4]	0	1.5‡	0.1	0.8†	0	0.9†	0	0.0§	0
Gastrointestinal										
Nausea	[13.1]	0.4	5.0†	0.5	4.8†	0.3	5.0†	1.3	5.8§	0
Gastric intolerance	4.8	0.4	4.2	0.3	3.5	0.2	5.7	0.8	2.5	0
Anorexia	[2.8]	0	0.0†	0	0.2‡	0	2.7	0	0.0	0
Vomiting	[7.2]	0	2.3†	0	0.8†	0	2.1†	0.2	0.4†	0.4
Diarrhea	[2.6]	0	0.5‡	0	0.8§	0	0.3†	0.1	0.0	0
Miscellaneous¶	4	0.3	6	0.7	5	0.8	16	1.6	0	0
Total discontinued		4.9†		2.6†		3.3†		[18.0]		2.5†

*Nos. = % of patients with side effects, boxed Nos. = the highest incidence per adverse reaction, and †, ‡, and § = significant differences from the highest incidence, ie, boxed number.

†$P < 0.0001$ (χ^2 test, Siegel S. Nonparametric Statistics, New York, McGraw-Hill Book Co, 1956, pp 104–111).

‡$P < 0.001$.

§$P < 0.01$.

¶ Various isolated instances of less relevant conditions.

TABLE 4.—Prevalence of the HLA A and B antigens in patients with hematologic changes

		Patients with agranulocytosis										
	European control population* (%)	RA† (16 patients)		SLE† (3 patients)	JRA† (1 patient)	AS, Reiter† (3 patients)	Other diseases† (3 patients)	Cancer (2 patients)	All cases (28 cases)		Patients with leukopenia (5 patients)	Patient with thrombocytopenia (1 patient)
		No. of patients	%	No. of patients	No. of patients	No. of patients	No. of patients	No. of patients	No. of patients	%		
A1	31	6	38	1		1		1	9	33	1	1
A2	48	9	56			1	2	1	13	48	1	
A3	28	4	25	1	1				6	22	2	
A9	17	1	6			1			2		1	1
AW23	4						1		1	19		
AW24	14	1	6	1					2			
A10	12	2	13						2			
AW25	4										1	
AW26	8	1	6						1	18	1	
AW34											1	
A11	12	1	6			1	1		3	11		
AW19							1		1			
A29	8											
AW30	4			1					1			
AW31	2						1		1	11	1	
AW32	8											
AW33	4										1	
A28	8	2	13						2	7		
AW36												
AW43												
Single A		5		1	1			2	9	33		
B5	12	3	19			1			4	15	1	
B7	24	4	25	2					6	22	1	
B8	21	4	25			1		1	6	22	1	1
B12	29	2	13				2	1	5	19	2	
B13	4	1	6						1	4		
B14	8										1	
BW15	12	2	13						2	7		
BW16	8											
BW38												
BW39		1	6						1	4		
BW17	8	1	6						1	4	1	
B18	10						1		1	4		
BW21	4										1	
BW22	6	2	13						2	7		
B27	8	8	50	1	(1)	(2)	2	1	12‡	50§		1
BW35	19	1	6	1	1		1		4			
BW37												
BW40	12	1	6	1					2	7	1	
BW41												
BW42												
Single B		2		1				1	4	15	1	

**From* HLA and Disease (Dausset J, and Svejgaard A, eds). Munksgaard, Williams and Wilkins Co, 1977, p 11.

†RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; JRA = juvenile rheumatoid arthritis; AS, Reiter = ankylosing spondylitis, Reiter; other diseases = mucocutaneous candidosis, psoriasis, warts (1 case each).

‡Excluding 3 cases of HLA-associated diseases.

§Significantly more than the expected 8% ($P = 0.0034$, χ^2 method).

TABLE 5.—HLA B27, sex, and rheumatoid factor of patients with rheumatoid arthritis and agranulocytosis

	B27 +		B27 –		B27 ?		All cases (total = 37)	
	Female	Male	Female	Male	Female	Male	Female	Male
Rheumatoid factor +	4	3	5	1	10	3	19	7
Rheumatoid factor –	0	0	2	0	2	0	4	0
Rheumatoid factor ?	1	0	0	0	6	0	7	0
Totals	5	3	7	1	18	3	30	7

arthritis, and acute uveitis (6). Omitting patients with B27-associated diseases, 12 of 24 patients with agranulocytosis in whom the HLA pattern was studied were B27 positive. This is significantly more than the expected 8%.

Despite the strong relationship between B27 and agranulocytosis, the incidence of this side effect is apparently not higher in ankylosing spondylitis than in rheumatoid arthritis. Patients with ankylosing spondylitis are predominantly male and rheumatoid-factor negative. Most are B27 positive. The relationship between B27, rheumatoid factor, and sex of patients with rheumatoid arthritis who develop agranulocytosis is shown in table 5. The patients were predominantly female (81%, which is more than usual in rheumatoid arthritis). None of the B27-positive patients was male and seronegative.

Agranulocytosis occurred with all doses and treatment regimens used (from 50 to 200 mg and from 3 days' treatment every other week to continuous treatment). Most patients had no previous history of blood dyscrasia or allergic reactions to other drugs. Agranulocytosis was usually not accompanied or preceded by rash, eosinophilia, or symptoms of Sjögren's syndrome.

There is no evidence that a particular type of concomitant medication predisposes to agranulocytosis. Eight patients had received levamisole alone. Most patients with rheumatic diseases received steroid or nonsteroidal anti-inflammatory drugs, while most cancer patients received cytostatics or radiotherapy. Nevertheless, experience in three leukemic children and in one patient with lung cancer suggests that levamisole may potentiate the leukotoxic effect of cytostatics if given simultaneously with these agents (not if alternated with such agents).

The leukocyte and neutrophil counts remained normal in nearly all of the patients studied until agranulocytosis was diagnosed; it occurred quite suddenly. One half of the cases occurred within the first 3 months of treatment. Some occurred after > 1 year. The onset seemed to be earlier after daily treatment than after intermittent treatment (fig 1).

In 81% of the patients, agranulocytosis was accompanied by an influenza-like illness with a general feeling of malaise, fever and chills, or other nonspecific symptoms. The blood dyscrasia was asymptomatic in only 19% of the patients.

Agranulocytosis was sometimes accompanied by a transient reduction in erythrocyte or platelet counts. This seemed more frequent in cancer patients than in patients with other diseases (table 6).

Figure 2 shows the restoration of leukocyte and neutrophil counts when levamisole treatment is discontinued. Numbers refer to the number of observations on which mean values are based.

The mean leukocyte and granulocyte counts gradually normalize after discontinuation of treatment, to reach mean values of 3000 and 1000/mm^3 respectively after 1 week. Many immature neutrophils appear in the peripheral blood. A temporary leukocytosis may be seen after recovery.

The bone marrow was examined in 49 patients. It is hypocellular during agranulocytosis and gives the impression of a maturation arrest at the promyelocyte stage. Erythrocyte, megakaryocyte, and lymphocyte counts are usually normal. During recovery, usually within 1 week, the marrow is hyperreactive. There is hyperplasia of the myeloid series which shows a shift to the left and good maturation. The bone marrow picture is indicative of allergic agranulocytosis. The time required for marrow recovery is that described for other drug-related neutropenias (7).

Some investigators have claimed that corticoste-

TABLE 6.—Concomitant hematologic changes

	Erythrocytes		Thrombocytes	
	Examined	Reduced	Examined	Reduced
Cancer	3	2	8	5
Other diseases	26	3	39	9

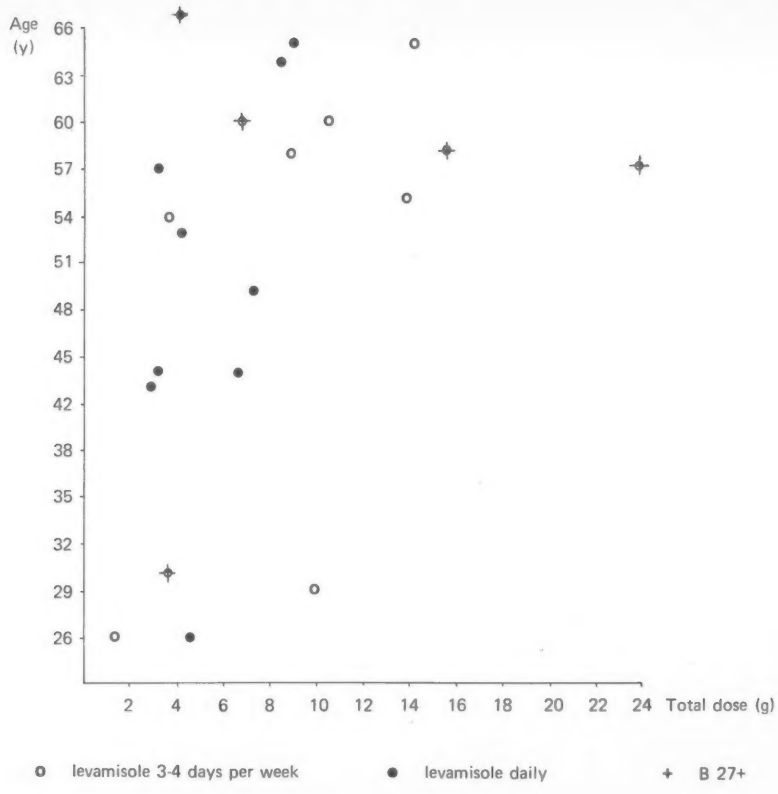

FIGURE 1.—Onset of agranulocytosis in patients with rheumatoid arthritis.

roids or granulocyte transfusions may be indicated to hasten recovery. In fact, 29 patients recovered spontaneously and uneventfully without corticosteroids and 35 patients recovered without blood or granulocyte transfusions (table 7). Of eight patients who died, at least four received corticosteroids and at least two received blood or granulocyte transfusions.

In all known cases, including the patients who died, blood counts rose after discontinuation of treatment. The deaths were due to late diagnosis and to overwhelming infection. There is no evidence that corticosteroids or prophylactic granulocyte transfusions hasten recovery. High doses of corticosteroids are not recommended because these may reduce host defenses and increase the risk of infection.

Eight patients who had experienced agranulocytosis during levamisole treatment were rechallenged with the drug. Three of these patients resumed the usual treatment on several consecutive days each week and developed a recurrence of agranulocytosis. They recovered upon withdrawal of the drug. Five other patients were rechallenged with one single dose of levamisole. Leukopenia followed within hours, as shown in figure 3, and lasted 1 or 2 days only. The appearance of young neutrophils indicates an immediate release of bone marrow reserves. These data suggest that the duration of agranulocytosis may be reduced by avoiding treatment on consecutive days.

Leukopenia

We have also examined 43 cases of leukopenia. This condition seems to differ from agranulocytosis in a number of ways (table 8).

Leukopenia is defined as a reduction of the number of leukocytes to $< 3000/mm^3$, with or without reduced neutrophil numbers. During agranulocytosis, neutrophil counts are always near 0.

The incidence of the HLA B27 antigen was not increased in patients with leukopenia, the bone marrow showed no changes, and flu-like or other

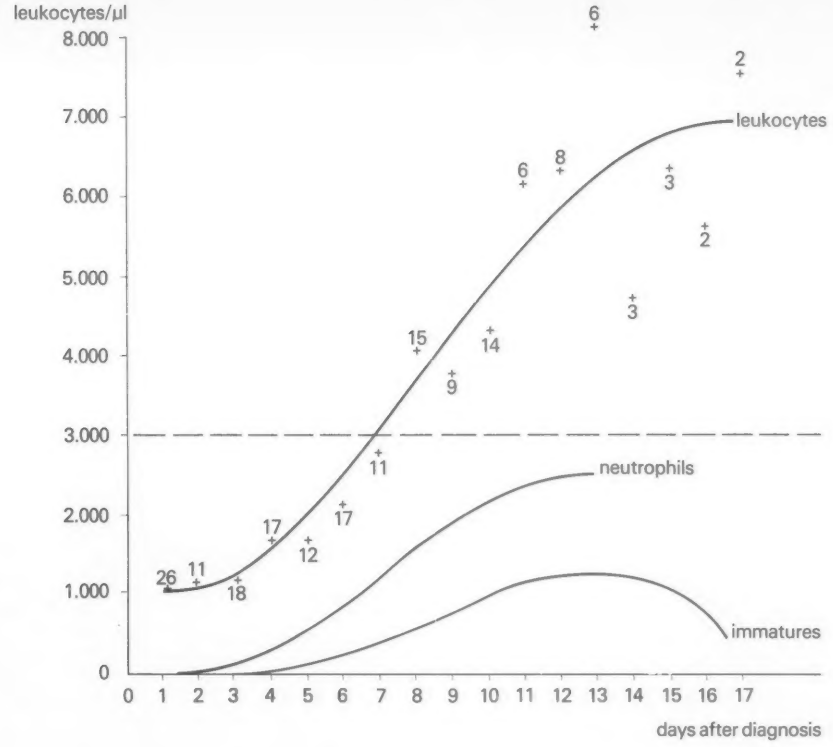

FIGURE 2.—Leukocyte counts after agranulocytosis (mean values).

symptoms were absent.When levamisole treatment was continued, leukopenia did not develop into agranulocytosis and the leukocyte count rose to normal. Leukopenia is therefore not an indication to discontinue treatment.

Thrombocytopenia

Three patients had thrombocytopenia (< 50,000 platelets/mm^3), which was associated with leukopenia in one patient. Neutrophil counts were normal. Two of the patients had rheumatoid arthritis. One of them, in whom HLA was determined, was B27 positive (table 4). All three cases occurred after 1–9 weeks of daily treatment. One patient had a moderate relapse (87,000 platelets/mm^3) 1 month after treatment was resumed.

MECHANISMS INDUCING ADVERSE REACTIONS

Little is known regarding the mechanisms inducing adverse reactions during levamisole treatment. Some reactions seem to have an immunologic basis. Other reactions seem to be temporary exacerbations of the underlying disease.

There is some indication that agranulocytosis is due to a type II reaction (Coombs' classification). Antibodies which agglutinate patient and/or donor neutrophils in the presence of levamisole have been found in the serum of four of eight patients tested. It is possible that levamisole acts as a hapten on the leukocyte membrane in sensitized patients, causing destruction of peripheral neutrophils. The regenerating capacity of the bone marrow remains intact and spontaneous recovery occurs when treatment is discontinued.

Leukopenia seems to have a different mechanism. It is more frequent with levamisole than with placebo, but granulocytes are not selectively involved and the leukocyte count is restored despite continuation of treatment. Leukopenia is usually not prodromal to agranulocytosis.

Febrile illness may be mild and transient or severe and progressive. A febrile episode may occur within hours after each intake of levamisole. It may

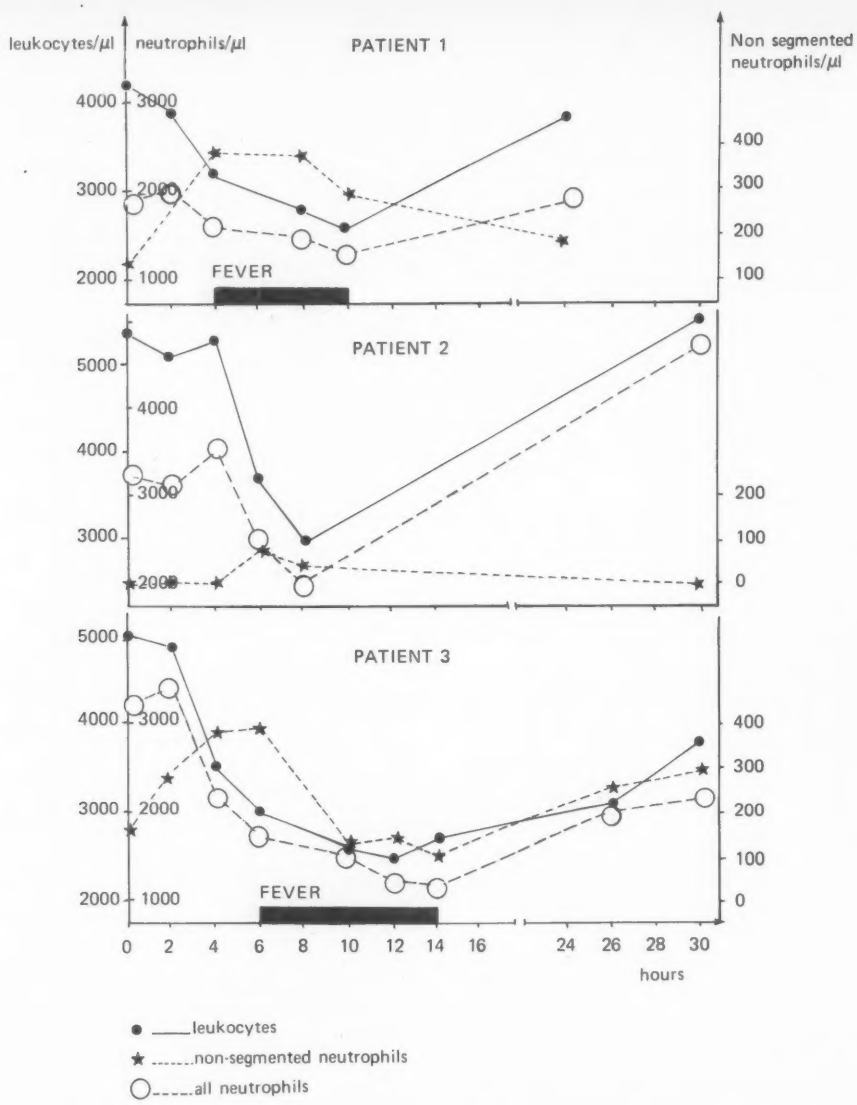

FIGURE 3.—Challenge with 1 × 150 mg of levamisole in 3 sensitized patients (*from* Veys E, and Mielants M. J Rheumatol 5:31–41, 1978).

be associated with chills, headache, malaise, myalgia, arthralgia, or other symptoms suggestive of influenza. Wbc counts may be decreased but often they are normal. Febrile illness may thus be a symptom of agranulocytosis, but often it is not. The indirect mast cell degranulation test in one patient with a febrile illness suggests that this patient had a type I allergy to levamisole (8).

Certain rashes also seem to be anaphylactic type I reactions. Their severity increases with repeated treatment courses and IgE is increased. Other rashes are accompanied by vasculitis and histologically resemble type III reactions (9,10).

Some adverse reactions resemble well-recognized complications of the primary disease. Fever and chills are not uncommon in autoimmune and neoplastic diseases. Arthritis may be a symptom of Crohn's or Behçet's disease. Vasculitis is an extra-articular manifestation of rheumatoid arthritis. Transient exacerbation of disease, which may occur

TABLE 7.—Treatment of agranulocytosis (88 patients)

	Recovered (50 patients)	Died* (8 patients)
Corticosteroids		
Yes	17	4
No	29	2
?	4	2
Transfusions		
Yes	13	3
No	35	3
?	2	2

*Cancer (3 patients); rheumatoid arthritis (2 patients); juvenile rheumatoid arthritis (2 patients); Hamman-Rich syndrome (1 patient).

TABLE 8.—Differences between agranulocytosis and leukopenia

	Agranulocytosis	Leukopenia
Wbc count	< 2000 cells/mm³	< 3000 cells/mm³
Neutrophils	< 20%	< 50%; > 20%
HLA B27 incidence	Increased	Normal
Bone marrow	"Maturation arrest" or hyperreactive	Normal
Symptoms	Generally present	Absent
Continued levamisole treatment	Persisting agranulocytosis	Restoration of leukocyte count
Challenge	Positive	Negative

during the first months of treatment with levamisole, may herald a good clinical response. Sometimes lesions reappear which have been dormant for several years. It is possible that levamisole produces such disease-related complications by unmasking pre-existing but latent immunologic abnormalities, rather than by initiating pathology (10,11).[5]

It is not known why idiosyncratic reactions to levamisole are more frequent in patients with rheumatic diseases than in other patients. Allergic reactions to other drugs also occur more frequently in patients with rheumatic disorders (12). D-Penicillamine, for example, is well-tolerated in Wilson's disease but causes many and severe adverse reactions in rheumatoid arthritis (13).

GENERAL CONCLUSIONS

It is evident that levamisole is well-tolerated by 80%–95% of the patients treated. Occasional reports on a higher incidence of side effects are not confirmed by placebo-controlled studies.

Significant adverse reactions are agranulocytosis, rash, and febrile illness. These reactions only occur in a small subpopulation of patients. This subpopulation is larger in rheumatic than in other diseases. Levamisole does not seem to cause renal or hepatic pathology.

Some adverse reactions seem to be allergic in nature and require discontinuation of treatment. Other reactions suggest over-responsiveness to the therapeutic action of the drug in highly susceptible patients and are not necessarily an indication to discontinue treatment. All complications are reversible after discontinuation of the drug.

Agranulocytosis, which must be differentiated from leukopenia, is the only potentially dangerous side effect. It is most frequent in patients with rheumatic diseases, in women, and in HLA B27 genotypes. It tends to occur predominantly within the first 3 months of treatment. It is not dangerous per se because it is spontaneously reversible when treatment is discontinued. The golden rule in the management of levamisole-induced agranulocytosis is early diagnosis, definite discontinuation of treatment, and the prevention of infection during the critical period. Therapeutic manipulation should be limited to the treatment of possible infection. Corticosteroids or granulocyte transfusions do not seem to hasten recovery and might even be harmful. Because agranulocytosis is usually accompanied by sudden symptoms suggestive of influenza, patients should be warned to consult the physician who prescribed levamisole when such symptoms appear after levamisole intake. At present, such a warning is included in each levamisole package and it is felt to be the most effective way for early agranulocytosis detection.

Although the possibility of agranulocytosis causes a risk to patients receiving levamisole, the morbidity may be reduced by the close supervision of a physician. If used under these conditions, the potential benefit to the patient probably outweighs the hazards and justifies the use of levamisole. The smaller doses and less frequent administration currently under study may further reduce the risks and increase the benefits of the drug.

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Overview of Thymosin Activity

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SUMMARY

Our current research program centers around the biologic and chemical characterization of the family of polypeptides present in thymosin fraction 5. A system of nomenclature has been developed and the peptides are being systematically isolated and chemically characterized. Thymosin fraction 5 and its component parts influence a variety of lymphocyte properties including cyclic nucleotide levels, migration inhibitory factor production, T-dependent antibody production, and expression of certain surface markers. Thymosin is being used in clinical trials to investigate its effects on immunodeficiency diseases, malignant diseases, and autoimmune diseases.

[Cancer Treat Rep 62:1731–1737, 1978]

The anatomic presence of the thymus gland in higher vertebrates and its marked involution at puberty has been known and described since the time of Galen (1). Yet it was not until 1961 that the thymus was shown to be the central organ of developmental mammalian immunity. Beginning with the studies of Miller (2) and Good et al (3), the necessity of an intact functional thymus for normal development of the immune response has been firmly established. Subsequent to the discovery of its ontogenic function, studies by Osoba and Miller (4) demonstrated that there was a humoral component of the thymus that could confer immune competence. This discovery marked the beginning of the study of thymic endocrinology.

Beginning with the purification of a preparation of rat thymus extract by Roberts and White in 1949, the isolation of thymic factors has been pursued by a number of investigators (5). However, it was not until the "rediscovery" of the thymus in 1961 that attempts to purify and characterize thymic factors began in earnest. One of the first preparations reported which demonstrated biologic (lymphocytopoietic) activity was the bovine thymus extract named thymosin (6). The initial characterizations of thymosin presupposed that the hormonal factors in the thymus were proteinaceous in nature, and purification proceeded on that basis (7). Subsequent characterization has, to date, supported that assumption.

In this paper we will describe progress currently being made in the characterization of the chemical composition and biologic activities of thymosin peptides. We will also summarize the ongoing clinical trials with thymosin in children with primary immunodeficiency diseases, in patients with cancer, and in patients with autoimmune diseases.

PROPERTIES OF THYMOSIN PEPTIDES

Biochemical Properties

Figure 1 indicates that thymosin fraction 5 contains a family of small acidic polypeptides with molecular weights ranging from 1000 to 15,000. Current studies suggest that biologic potency of fraction 5 may be due to several of these peptides active either together or individually on various subpopulations of T cells. In order to facilitate the identification and comparison of thymic peptides from one laboratory to another, we have suggested a nomenclature for the identification of each of the

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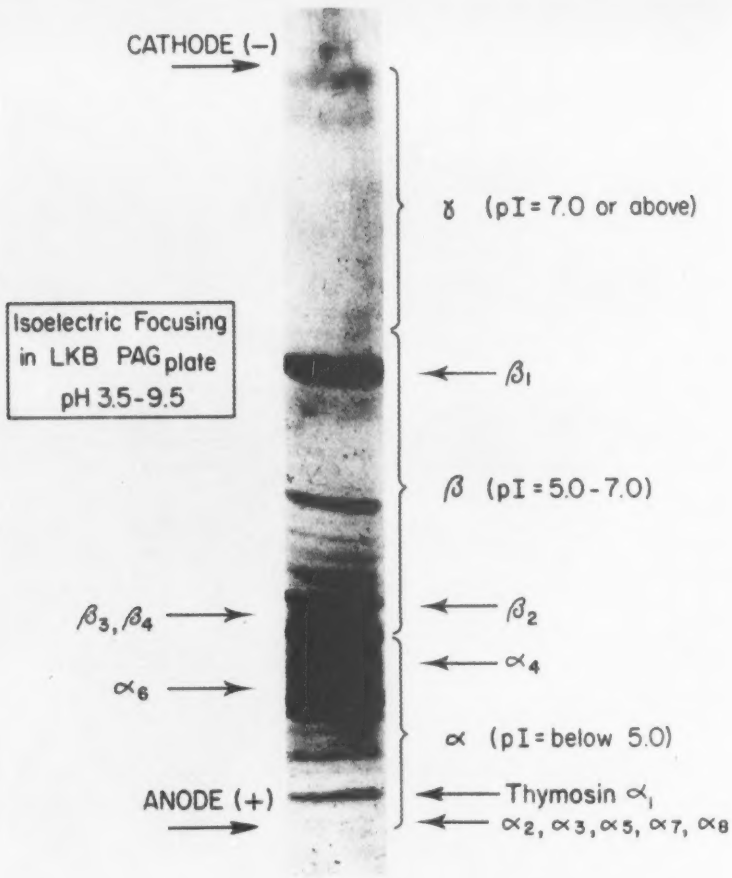

FIGURE 1.—Thymosin fraction 5 contains a family of small acidic polypeptides with molecular weights ranging from 1000 to 15,000.

peptides based upon the isoelectric focusing pattern of thymosin fraction 5 in the pH range of 3.5–9.5 (8). Peptides are divided into three major regions based upon their migration patterns. The regions are identified as alpha (α), beta (β), and gamma (γ). The α region consists of the peptides with isoelectric points (pI) < 5.0 (highly acidic), the β region consists of peptides with pI between 5.0 and 7.0 (acidic), and the γ region consists of peptides with pI > 7.0 (basic). Sequential numbering is used to identify peptides from each region in the order of their isolation. As can be seen in figure 2, we have isolated and purified 12 of the polypeptides to homogeneity or near homogeneity. Eight of the peptides are from the α region and four are from the β region. Two of the peptides have now been sequenced, and primary structures of several others are currently being studied. Thymosin α_1, the first of the biologically active peptides isolated, has 28 amino acid residues and a molecular weight of 3108. The sequence of this peptide is shown in figure 3. Thymosin α_1 is from ten to 1000 times as active as thymosin fraction 5 in a number of functional in vitro and in vivo T-cell assays (8). Recently, Drs. Meienhofer and Wang at Hoffmann-La Roche, Inc (Nutley, NJ), have synthesized thymosin α_1 (Meienhofer, personal communication). Preliminary studies indicate that the synthetic peptide is as biologically active as the natural α_1. The β_1 peptide is biologically inactive and is presumed to be carried through the purification scheme as a contaminant. The sequence of β_1 has been found to be homologous to

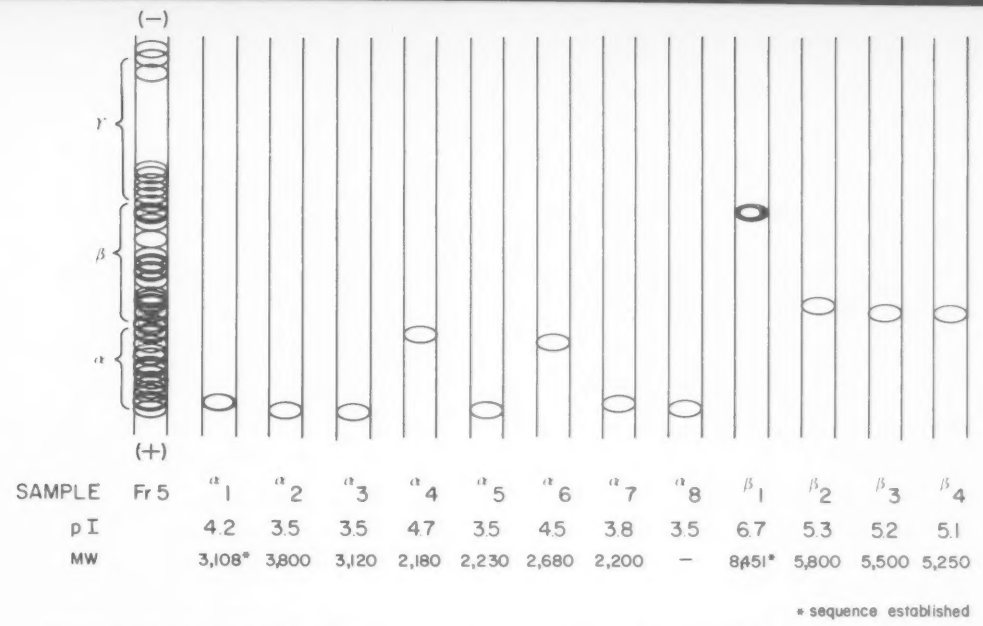

FIGURE 2.—12 polypeptides isolated and purified to homogeneity or near homogeneity from thymosin fraction 5.

the N-terminal 37 amino acid residues of A-24, a nonhistone chromosomal protein originally described by Olson et al (9) and later by Schlesinger et al (10) who named it ubiquitin.

Biologic Activities

Thymosin-induced changes in cyclic nucleotide levels.—One of the first detectable effects of thymosin fraction 5 on thymocytes is to increase intracellular cyclic GMP levels (11). Using a radioimmunoassay (RIA) for the determination of cyclic AMP and an acetylation RIA procedure to measure cyclic GMP, we have observed that in vitro incubation with thymosin causes a significant increase in cyclic GMP levels, but not cyclic AMP levels, in murine thymocytes (table 1). In contrast to our findings with thymosin, Kook and Trainin using thymic humoral factor (12) and Scheid et al (13) using thymopoietin and ubiquitin have reported that these thymic factors increase cyclic AMP levels. These contrasting results support the hypothesis that a family of thymic peptides exists whose influence over T-cell development could be via several different mechanisms which may include changes in cyclic GMP or cyclic AMP or possibly other mechanisms which may not directly involve cyclic nucleotides in their mode of action.

Thymosin-induced changes in lymphocyte function.—Field and Shenton (14) have reported that thymectomy of guinea pigs that had been sensitized 10 days before to tuberculin purified protein derivative (PPD) causes a rapid loss of the reactivity of peripheral blood lymphocytes to PPD as measured by a macrophage electrophoretic mobility test. The reactivity could be restored in vitro by the addition of small amounts of serum from thymus-bearing guinea pigs and also by human serum. Serum from thymectomized animals could not restore reactivity of the peripheral blood lymphocytes to PPD. They suggested that the substance in the normal serum which allows for reconstitution of this system is thymosin, although thymosin was not utilized in their system. Based upon their observations, we have developed an assay for studying the effects of thymosin and purified thymosin peptides on migration inhibitory factor (MIF) in vitro as illustrated in figure 4. Thymosin fraction 5 can restore a thymectomized guinea pig's response to PPD. The addition of as little as 10 ng/ml of thymosin α_1 has induced cells capable of MIF production, while thymosin α_3 at concentrations as high as 1000 ng/ml does not induce MIF-producing cells. We are also developing an assay to measure the effect of thymosin on plaque-forming cells in vitro using a modified Mishell-Dutton procedure with normal, thymectomized, and sham-operated animals. Thymosin fraction 5, at a concentration of 100 μg/ml in tissue culture, stimulates an increase in plaque-forming cells of normal and sham-thymectomized mice. A similar

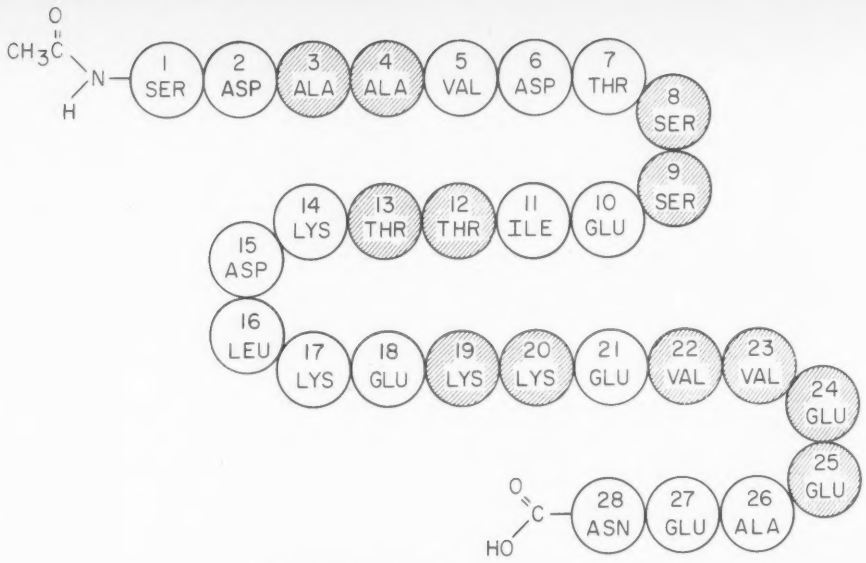

FIGURE 3.—Sequence of thymosin α_1.

preparation of spleen fraction 5 stimulates in a similar fashion. It is not presently understood why a spleen fraction 5 is stimulatory to lymphocytes derived from mice with intact thymus glands. However, following adult thymectomy (> 2 weeks after thymectomy), the capacity of thymosin fraction 5 to stimulate this increase is significantly enhanced whereas the capacity of spleen fraction 5 is somewhat reduced. We have recently found that the hapten dinitrophenol (DNP) conjugated to thymosin fraction 5 can enable nude mice to produce anti-DNP antibodies. However, DNP conjugated to ovalbumin did not produce anti-DNP antibodies in nude mice (15). Figure 5 diagrams a postulated mechanism for this thymosin effect. We are investigating the possibility that nude mouse pre-T cells may have thymosin receptors which bind thymosin molecules. This binding event could induce or mature the pre-T cell to produce a "helper factor(s)" which may act on the B cells to allow them to produce antibodies.

Thymosin-induced expression of surface markers.—Current experimental data indicate that thymosin peptides can induce the expression of various surface markers on lymphoid cells from different sources. Attempts at characterization of T-lymphocyte subpopulations by their expression of different antigens of the Lyt system are currently being investigated (*see* Ahmed et al, this issue). We are also investigating the expression of enzymes in bone marrow cells after incubation with thymosin in vitro. Terminal deoxyribonucleotidyl transferase (TdT) is normally present in thymocytes but not in certain bone marrow cell fractions or spleen cells (16). Thymosin fraction 5 can induce the expression of TdT in murine bone marrow cells. The function of this enzyme is presently unknown.

TABLE 1.—Cyclic nucleotide levels determined after 15 mins of incubation at 37°C with the agents indicated

	Cyclic AMP (pmols/10^7 cells)	Cyclic GMP (fmols/10^6 cells)
Medium control*	1.70 ± 0.17†	2.70 ± 0.63
100 μg of thymosin fraction 5	1.38 ± 0.03 (NS)‡	7.55 ± 0.07 ($P < 0.001$)
100 μg of spleen fraction 5	0.81 ± 0.03	1.57 ± 0.21 (NS)

*RPMI-1640.
†SD for independent triplicate samples.
‡Statistical significance based on Student's t test; NS = not significant, $P > 0.05$.

CLINICAL EXPERIENCE WITH THYMOSIN

One of the most important clinical potentials of thymosin is its possible relationship to the management of thymic-dependent diseases. Many such thymic-dependent conditions are known, such as immunodeficiency diseases and certain autoimmune diseases; other relationships are suspected, such as cancer.

All thymosin fraction 5 used in our clinical studies was prepared as previously described (7). The first 60 patients in our clinical studies were treated with thymosin fraction 5 prepared in our laboratories.

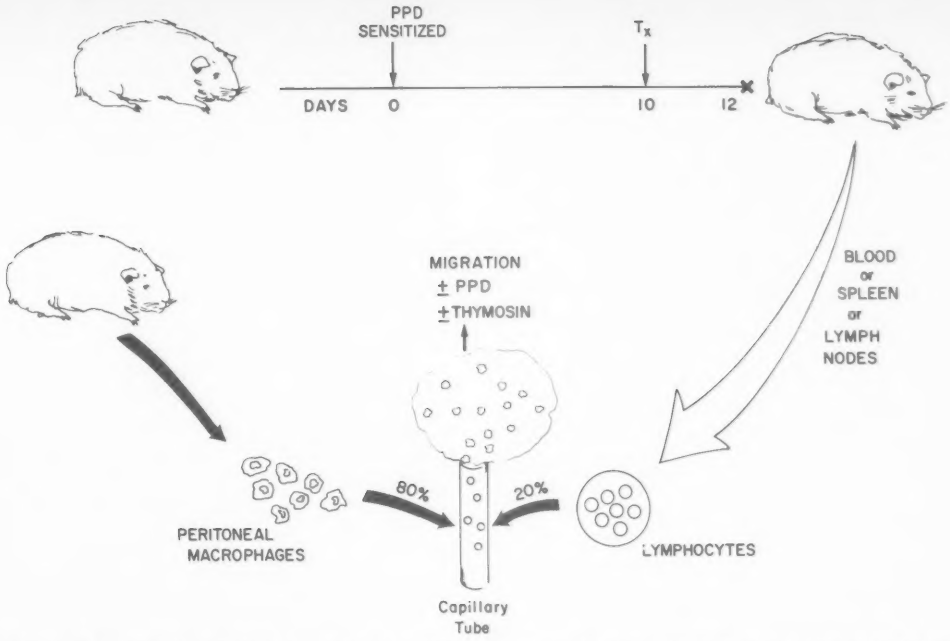

FIGURE 4.—Assay for studying effects of thymosin and purified thymosin peptides on MIF production in vitro using guinea pig lymphocytes.

This preparation met FDA standards for safety, sterility, and nonpyrogenicity. Since January 1976, all of the clinical quality thymosin used has been prepared by Hoffmann-La Roche, Inc.

One of the earliest uses of thymosin in human research was a study of the effect of thymosin in vitro on spontaneous E rosette levels of human peripheral blood cells (17). Subsequently, thymosin has been used in phase I and II protocols in patients with primary immunodeficiency diseases, patients with cancer, and patients with certain autoimmune diseases. In the phase I trials, no evidence of central nervous system, liver, kidney, or bone marrow toxicity has been seen (18). Eighty percent of the pediatric patients who have demonstrated increased in vitro E rosettes with thymosin have also responded in vivo with increased T-cell numbers and function. A substantial number of cancer patients

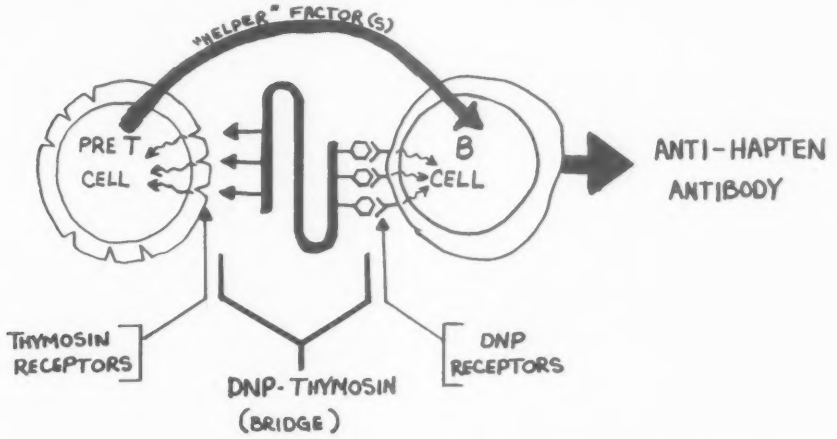

FIGURE 5.—Diagram of a postulated mechanism for the action of DNP-conjugated thymosin in nude mice.

in the phase I trials have responded in similar fashion. Additional phase II studies are in progress or planned at several cancer centers to determine the efficacy of thymosin administered to cancer patients in combination with other therapeutic modalities (19).

The first efficacy trial (phase II) of thymosin has now been completed in nonresectable small cell carcinoma of the lungs by Dr. Paul B. Chretien and his associates at the National Cancer Institute (*see* Chretien et al, this issue). Of major importance in this trial was the finding that thymosin, when given in conjunction with intensive chemotherapy, could increase the mean survival time from 225 to 450 days.

More recently we have initiated phase I trials with thymosin in the treatment of autoimmune diseases. To date, five patients with autoimmune diseases have been treated with thymosin fraction 5 for periods ranging from 4 to 16 months.[5] Four of the patients had systemic lupus erythematosus and the fifth had severe rheumatoid arthritis and Sjogren's syndrome. In this preliminary open study there have been no ill effects related to the administration of thymosin and in three patients there has been objective immunologic and clinical improvement. Based upon these encouraging findings, a phase II randomized trial is planned to determine the efficacy of thymosin in systemic lupus erythematosus.

POSTULATED MECHANISM OF THYMOSIN ACTION

Based upon accumulated experimental evidence, thymosin appears to trigger maturational progression of several (possibly distinct) early stages of T-cell development and perhaps to augment the capacity of certain mature T cells to respond to antigens. Recent studies suggest that thymosin may act through a cyclic GMP second messenger (11) and may induce the expression of alloantigens on the surface of T cells as they develop their functional capacity for immunocompetence (20).

Thymosin can induce the expression of Lyt phenotypes and TdT in bone marrow cells (21). Furthermore, thymosin can regenerate suppressor cells in thymectomized mice who lose these cells soon after thymectomy (Marshall and Goldstein, manuscript in preparation). There is good evidence to propose that thymosin affects the very early stages of T-cell development, possibly by acting on pre-T cells. It is this property of thymosin that is thought to induce immune reconstitution in anergic individuals (fig 6). Therefore, it is the ability to induce functionally mature cells from functionally immature cells that may prove thymosin to be superior to other forms of immunologic therapeutic modalities such as adjuvant therapy which requires availability of relatively mature T cells. Thus, the range of thymosin activity (as outlined in fig 6) points to the usefulness of thymosin in reconstituting severely immunosuppressed patients, provided that they still have the necessary thymosin-sensitive precursor T-cell populations. Recent studies by Kenady and co-workers (22) provide support for this hypothesis in cancer patients. These investigators have observed that even after large doses of radiotherapy or chemotherapy, the peripheral blood of patients with many types of cancer still contains thymosin-sensitive lymphocytes.

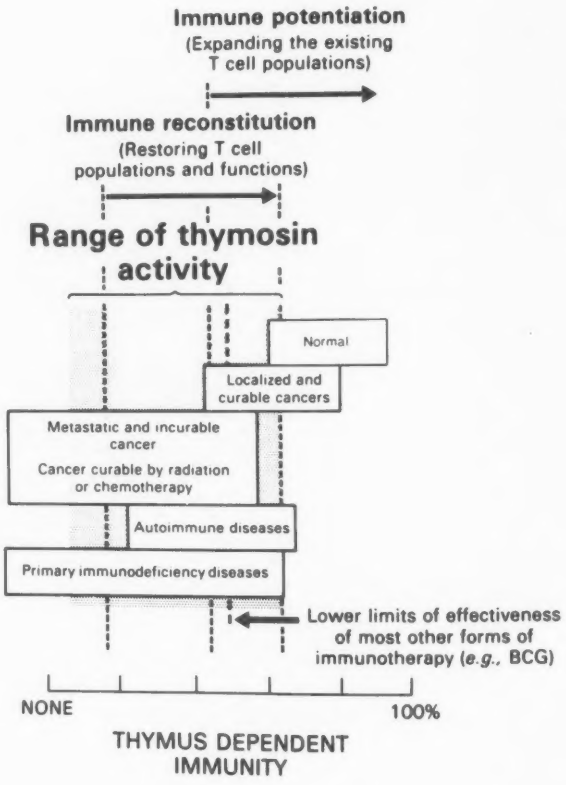

FIGURE 6.—Range of thymosin activity.

[5]Lavastida MT, Rossio JL, Goldstein AL, et al. Thymosin treatment in autoimmune disorders. Submitted for publication.

THYMOSIN THERAPY: POTENTIALS FOR THE FUTURE

As more data accumulate to indicate that a variety of diseases are thymic-dependent, it becomes clear that specific immunotherapeutic agents will be necessary to treat these specific immune defects. Thymosin offers a unique potential, in that it is a family of polypeptides with differing biologic capabilities. As the various fractions become more fully characterized, their selective biologic activities will be elucidated. An increased understanding of the mechanism of action of these peptides on T-cell subpopulations from patients with primary and secondary immunodeficiency diseases, certain cancers, and autoimmune diseases (whose etiology is associated with an immune imbalance) gives hope that one day thymosin may have routine use in the treatment of these diseases.

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In Vitro Induction of Lyt Surface Markers on Precursor Cells Incubated With Thymosin Polypeptides [1]

A. Ahmed,* A. H. Smith, D. M. Wong, G. B. Thurman, A. L. Goldstein, and K. W. Sell [2-4]

SUMMARY

Induction of precursor T cells by thymosin fraction 5 was found to generate a population of cells bearing the Lyt 1^+, 2^+, and 3^+ phenotype and functionally capable of providing T-cell helper activity in vitro. However, when the component polypeptides of thymosin fraction 5 were similarly analyzed, several displayed inductive activities quite different from those of the parent preparation. These results suggest that variations in the polypeptide composition determine, to a large part, the inductive properties of a thymosin preparation and that such variations in vivo may serve as a regulatory system for T-cell maturation.

[Cancer Treat Rep 62:1739–1747, 1978]

Several lines of evidence have suggested that extracts of thymus tissue induce the differentiation of precursor cells into cells that bear phenotypic resemblance to the thymus-derived (T) cells (1) and further maturation of cells can function as immunologically competent T cells (2). Such preparations include thymosin (3), thymic humoral factor (4), thymin or thymopoietin (5), and thymic factor (6). Studies on T-lymphocyte differentiation are greatly aided by surface markers which are unique to this population of lymphoid cells. These include the thymus-leukemia antigen (7), the theta antigen (thy-1) (8), and a series of antigens termed the Lyt cell-surface markers (9,10). Cytotoxic antisera specific for these T-cell surface markers, along with the availability of mice congenic for these markers, provided us a format for the study of in vitro phenotypic conversion of precursor cells initiated by thymosin and various preparations of polypeptides isolated from thymosin fraction 5 (fr. 5). Further, following phenotypic conversion, these cells were assayed in vitro for their functional maturation, as indicated by their ability to interact with syngeneic spleen cells in the formation of antibody to sheep red blood cells (SRBC). Results from these experiments form the basis of this report.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Me). C57BL/6-Ly 1^a and C57BL/6-Ly 2^a3^a mice were bred from breeding pairs kindly provided by Dr. M. Cherry, the Jackson Laboratory, and the athymic nu/nu mice and their +/nu littermates were obtained from the Division of Animal Resources, National Institutes of Health (Bethesda, Md) and other sources. Adult thymectomized, irradiated bone marrow-reconstituted mice (T × BM) were obtained as described previously (2).

[1]Supported by the Naval Medical Research and Development Command, Research Work Unit No. MR041.02.01.0034. The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Resources, National Research Council, DHEW Publication No. (NIH) 74–23.

[2]The following institutions and investigators participated in this study: Naval Medical Research Institute, Bethesda, Md (*A. Ahmed* and *D. M. Wong*); Georgetown University Medical School, Washington, DC (*A. H. Smith*); The University of Texas Medical Branch, Galveston (*G. B. Thurman* and *A. L. Goldstein*); and National Institute of Allergy and Infectious Diseases, Bethesda, Md (*K. W. Sell*).

[3]We are deeply grateful to Dr. M. Cherry of the Jackson Laboratory for the Ly congenic mice, Dr. B. J. Mathieson for advice regarding the use of anti-Ly sera, and Janie P. Kaczmarowski for her expert editorial assistance.

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Fractionation of Cells

Suspensions of bone marrow cells were obtained by the gentle flushing of the tibia from the hind legs with sterile media. These bone marrow cells were washed in media and applied on discontinuous bovine serum albumin (BSA) gradients, as described by Komura and Boyse (1). Four layers were obtained: A (at the 10%–23% interface), B (23%–26%), C (26%–29%), and D (29%–35%). Spleen cells were treated with isotonic-NH_4Cl for lysis of rbcs before separation on BSA gradients.

Media

Media used throughout these experiments consisted of RPMI-1640, supplemented with penicillin, streptomycin, L-glutamine, and 25 mM Hepes, pH 7.4. For Mishell-Dutton assays, the culture conditions outlined by Mishell and Dutton were employed (11).

Cytotoxicity

The cytotoxicity test was performed by the dye exclusion technique in microtiter trays, as previously described (12). The test was performed in triplicate and each value represents the mean percent of net cytotoxicity, net cytotoxicity being defined as total cytotoxicity minus the cytotoxicity seen with normal mouse serum and rabbit complement (C′). The C′ used was a preselected lot which showed minimum cytotoxicity against mouse lymphoid cells.

Source of Helper Cells

C57BL/6 mice were primed by an iv injection of 2×10^6 erythrocytes and 4 days later their spleens were removed. Splenic T cells were isolated by filtration through nylon wool columns, as described elsewhere (13).

Antisera

Hyperimmune antisera against Lyt cell surface markers were prepared, as described by Shen et al (14). The Lyt 1.1, 2.1, and 3.1 were absorbed with NZB thymocytes; the Lyt 1.2 and 2.2 were absorbed with DBA/2 thymocytes; and the Lyt 3.2 was absorbed with AKR thymocytes prior to use. This was found to be necessary for removing unwanted specificities against thymocyte surface viral antigens present in anti-Lyt cell sera (personal communication, B. J. Mathieson, National Institutes of Health).

Thymosin Fr. 5 and Its Polypeptides

The procedure for the preparation of thymosin fr. 5 has been described elsewhere (2). The isolation of the various polypeptides, α1, α5, and α7, is described in figure 1.

Mishell-Dutton Cultures

Spleen cells (1×10^7) from T × BM mice (thymectomized, irradiated, and reconstituted with anti-thy 1.2 + C′-treated bone marrow cells) were cultured in vitro in the presence or absence of 5×10^6 SRBC, as described by Mishell and Dutton (11), and assayed on Day 5 for total direct plaque-forming cells against SRBC. Assays were performed in triplicate and the mean ± SE of the direct plaque-forming cells per culture was calculated.

RESULTS AND DISCUSSION

Bone marrow cells from C57BL/6, C57BL/6-Ly 1^a, C57BL/6-Ly 2^a3^a T × BM, and C57BL/6 athymic nu/nu mice were separated into four fractions, A, B, C, and D, using discontinuous BSA gradients. A total of 2×10^6 cells from each layer were incubated in vitro for 90 minutes at 37°C with the various thymosin preparations. Following incubation, the cells were washed twice and assayed for acquisition of the Lyt cell-surface markers by their sensitivity to the cytotoxic anti-Lyt cell antisera in the presence of C′, as described in the Materials and Methods section. As seen in table 1, after incubation with 100 μg/ml of thymosin fr. 5, cells from layer A and particularly layer B of C57BL/6 T × BM and athymic nu/nu mice demonstrate the Lyt 1.2, 2.2, and 3.2 markers, whereas those from layers C and D do not display these surface antigens. Specificity of the antisera used in these experiments is shown by the fact that (*a*) anti-Lyt 1.1, 2.1, and 3.1 are not cytotoxic for these cells; (*b*) T × BM bone marrow cells from layers A and B of C57BL/6-Ly 1^a mice, following incubation with 100 μg/ml of thymosin fr. 5, display the Lyt 1.1, 2.2, and 3.2 characteristics, but not Lyt 1.2, 2.1, and 3.1 characteristics; and (*c*) similarly, T × BM bone marrow cells from layers A and B of C57BL/6-Ly 2^a3^a mice, following incubation with 100 μg/ml of thymosin fr. 5, display the acquisition of Lyt 1.2, 2.1, and 3.1, but not Lyt 1.1, 2.2, and 3.2 surface antigens.

In addition to the results presented in table 1, it was found that the sequential action of αLyt 1 + C′, followed by treatment with αLyt 2 + C′, did not increase the cytotoxicity obtained by treatment with αLyt 1 + C′ alone. This indicates that the major phenotype of the induced cells was Lyt 1^+, 2^+,

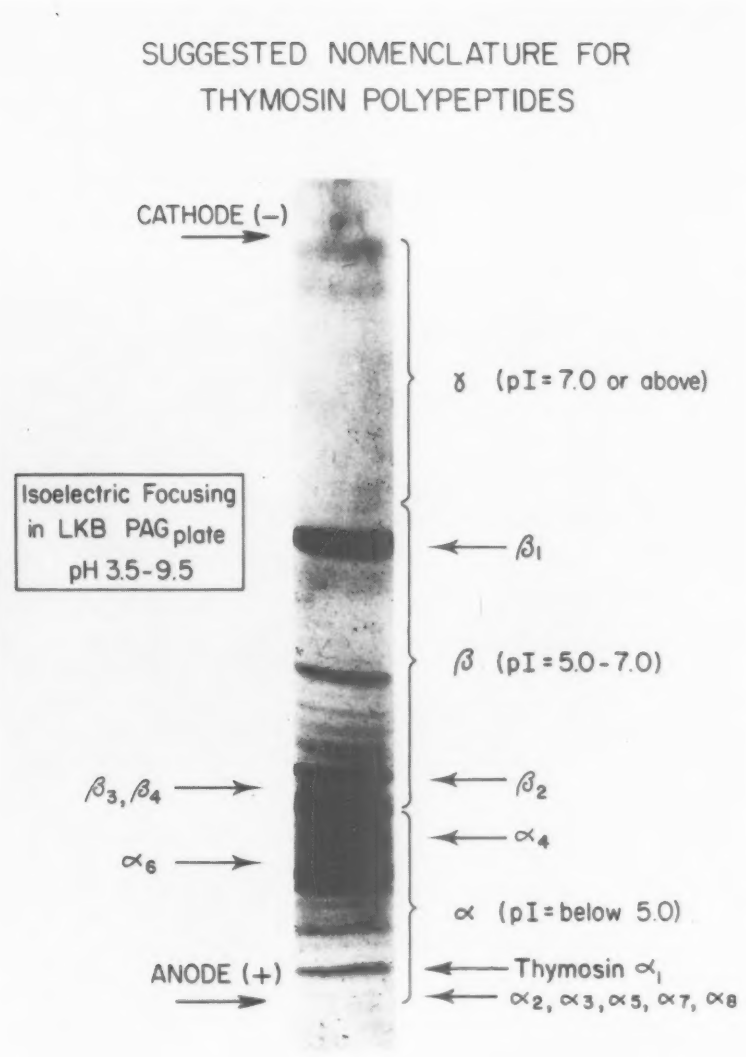

FIGURE 1.—Isoelectric focusing of thymosin polypeptides on LKB PAG-PLATE, pH 3.5–9.5.

TABLE 1.—Induction of Lyt surface markers by thymosin fr. 5 on BSA gradient-separated bone marrow cells from thymus-deprived mice*

Bone marrow from—	BSA gradient layer	% net cytotoxicity					
		Antisera specific for Lyt—					
		1.1	1.2	2.1	2.2	3.1	3.2
C57BL/6 T × BM (1.2, 2.2, 3.2)	A	1.3	7.4	1.9	6.1	2.6	6.9
	B	−2.7	25.8	1.7	18.7	3.7	19.3
	C	2.9	3.1	−4.8	4.1	2.1	3.4
	D	3.4	0.9	−3.6	3.3	3.7	1.1
C57BL/6-Ly 1^a T × BM (1.1, 2.2, 3.2)	A	2.8	1.9	3.3	4.9	4.3	7.6
	B	28.4	2.7	2.6	21.8	2.7	20.4
	C	9.3	−4.0	3.1	6.7	3.8	4.8
	D	4.4	−3.6	1.7	2.9	1.9	3.1
C57BL/6-Ly 2^a3^a T × BM (1.2, 2.1, 3.1)	A	−3.3	4.3	2.6	6.1	8.1	4.8
	B	−2.6	24.4	16.8	2.7	15.9	5.3
	C	3.7	8.6	5.3	−4.4	7.1	3.4
	D	2.1	4.9	1.8	−7.1	5.4	2.6
C57BL/6 nu/nu (1.2, 2.2, 3.2)	A	0.6	11.9	2.3	9.2	1.4	12.3
	B	3.4	26.1	4.1	27.2	3.6	25.8
	C	2.7	4.8	0.9	3.3	2.6	3.4
	D	1.3	3.4	1.6	5.1	2.8	−4.0

*Bone marrow cells (2×10^6) from each layer of BSA gradients were washed 2 times and then incubated for 4 hrs at 37°C with 100 μg/ml of thymosin fr. 5; the net cytotoxicity was determined as described in the Materials and Methods section.

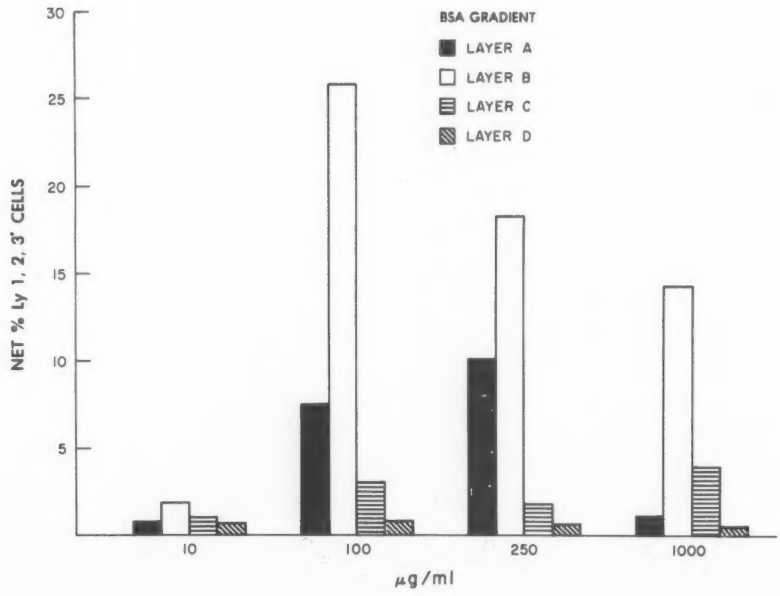

FIGURE 2.—Induction of Lyt phenotype as a function of thymosin concentration. Bone marrow cells from nu/nu mice were fractionated on BSA gradients and 2×10^6 cells from each gradient layer were incubated in 1 ml of the indicated concentration of thymosin fr. 5 for 90 mins at 37°C. The Lyt phenotype was determined in microcytotoxicity assays.

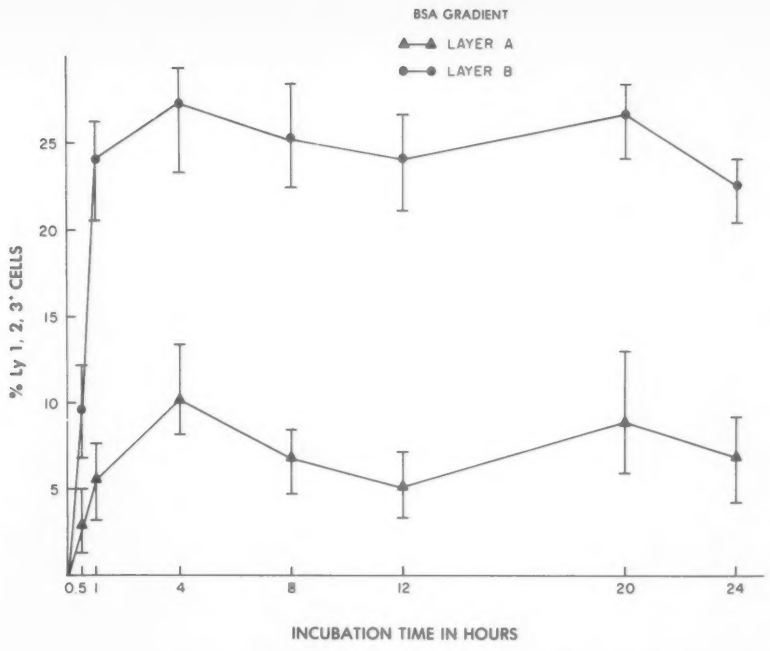

FIGURE 3.—Kinetics of Lyt phenotype conversion by thymosin fr. 5. Bone marrow cells from nu/nu mice were fractionated on BSA gradients and 2×10^6 cells from layers A and B (Lyt precursor-rich layers) were incubated in 1 ml of thymosin at 100 μg/ml at 37°C for the indicated time intervals, after which the Lyt phenotypes were determined in microcytotoxicity assays.

3^+ and that few, if any, of these cells displayed the Lyt 1^-, 2^+, or 3^+ phenotype.

A concentration of 100 μg/ml of thymosin fr. 5 for 2×10^6 bone marrow cells from layers A and B was found to give optimal induction of the relevant phenotype on precursor T cells based on initial experiments. Thus, as seen in figure 2, lower concentrations (10 μg/ml) or higher concentrations (250 μg/ml or 1 mg/ml) caused a significant decrease in the expression of Lyt cell-surface markers, denoting perhaps an inhibitory effect at higher concentrations. This inability of higher concentrations to induce Lyt expression, however, was not secondary to toxicity in the thymosin preparation. Figure 3 demonstrates the kinetics of the acquisition of Lyt cell-surface markers on layers A and B of bone marrow from T × BM mice. As seen, significant Lyt expression occurred within 1–4 hours of in vitro incubation of 2×10^6 precursor cells with 100 μg/ml of thymosin, and the frequency of the cells bearing these phenotypes remained constant even after incubation in vitro for up to 24 hours. Once again, layer B contained more precursor T cells than layer A. It is not known if this seemingly constant proportion of Lyt-bearing cells reflects a static population of Lyt-converted cells or if there is a continuous entry into and exit from the Lyt^+ pool. It is difficult to assess either possibility with the data available; however, use of metabolic inhibitors of protein and RNA synthesis may reveal whether such subtle changes occur.

In addition to studies of bone marrow cells, the occurrence of similar precursor cells in the spleens of normal mice was investigated. It was found, however, that data obtained with spleen cells from adult mice (4–6 weeks of age) were inconsistent, due perhaps to an age-dependent loss of the precursor cells (data not shown). Therefore, experiments were carried out with spleen cells from nu/nu C57BL/6 mice of varying ages, separated on BSA gradients into layers A, B, C, and D. It was found (fig 4) that spleen cells from 7–14-day-old mice contained the highest frequency of precursor T cells, after which there was an age-related, gradual loss in these precursor cells. Spleen cells from adult T × BM mice, however, always showed a profile similar to that seen with 7–14-day-old athymic nu/nu bone marrow cells. These data suggest that a radiosensitive, non-T-regulatory cell controls the level of Lyt precursor cells in the adult spleen.

Since thymosin fr. 5 consists of several polypeptides, efforts were made to delineate which polypep-

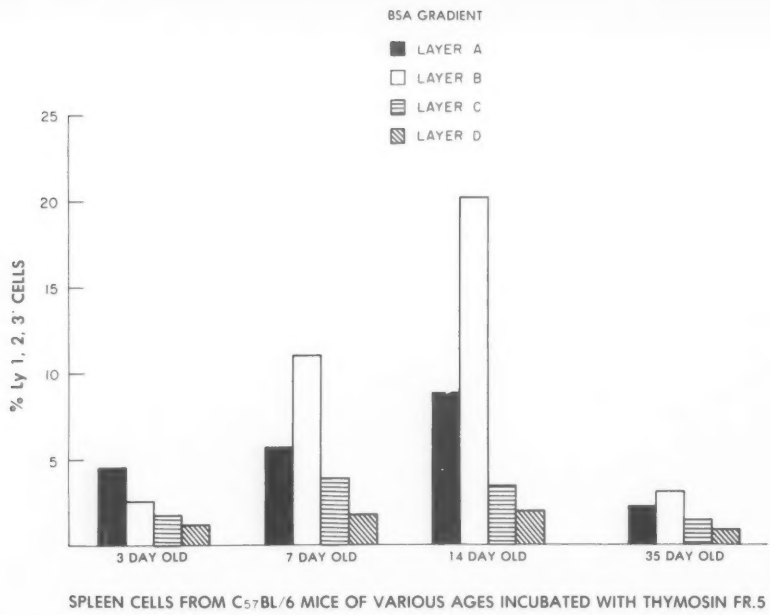

FIGURE 4.—Variation in splenic Lyt precursor cell frequency with age. Spleen cells from C57BL/6 nu/nu mice were fractionated on BSA gradients and 2×10^6 cells from each layer were incubated in 1 ml with 100 μg of thymosin fr. 5 for 90 mins at 37°C. Subsequently, the cells were analyzed for the frequency of Lyt precursor cells, as denoted by a net increase in Ly^+ cells over control cultures.

tides were active in these inductive events. Bone marrow from T × BM C57BL/6 mice was fractionated into layers A, B, C, and D and subsequently incubated in vitro with various concentrations of α1, α5, and α7. As seen in figure 5, both α1 and α7 were able to induce the expression of Lyt phenotypes, but α5 was not. These data indicated that more than one thymic polypeptide could stimulate the inductive events related to the acquisition of surface Lyt surface markers, and that there were other thymic polypeptides which could act as inhibitors of the inductive process. This suggests that changes in the relative proportion of these polypeptides is one explanation for differences observed in the various biologic assays of thymosin.

Further, functional assays were performed with these in vitro-induced cells in an effort to relate the acquisition of surface membrane T-cell characteristics with immune function. Thus, bone marrow cells (1×10^6) from T × BM mice separated into layers A, B, C, and D on BSA gradients were cultured in vitro with syngeneic T × BM spleen cells (7.5×10^6) in the presence or absence of SRBC. As seen in table 2, while no measurable response was detected in T × BM spleen cells cultured with bone marrow cells from untreated layers, following incubation with 100 μg/ml of thymosin fr. 5, cells from layers A and B were able to induce helper activity. These data indicate that functional helper T-cell activity can be induced by thymosin fr. 5 and that the inducible helper T-cell precursors partition on the BSA gradient in a similar pattern to the Lyt^+ cell precursors.

When similar experiments were carried out with polypeptide α7-treated bone marrow cells from layers A, B, C, and D, no such helper activity was seen (table 3). However, coculture with α7-treated layer B cells caused a profound suppression of the ability of helper cells to induce an antibody response to SRBC. This phenomenon was further investigated by the treatment of the α7-treated cells with the various anti-Lyt sera. As seen in table 4, this suppressive effect of α7-incubated bone marrow cells on the in vitro capacity of primed helper T cells to induce an antibody response was susceptible to lysis with all three anti-Lyt sera, Lyt 1.2, 2.2, and 3.2.

These data, plus the previous observation that few if any Lyt 1^-, 2^+, or 3^+ cells are induced by thymosin fr. 5, suggest that α7 induces a suppressor cell population which is Lyt 1^+, 2^+, and 3^+. Although suppressor T cells were initially phenotyped Lyt 1^-,

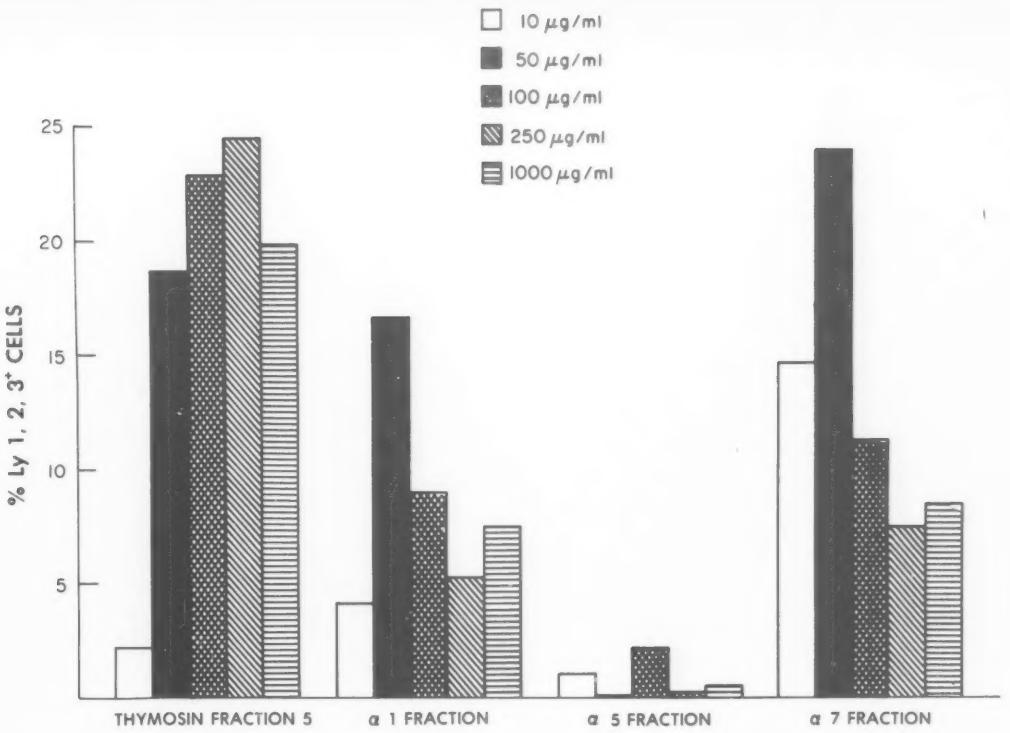

FIGURE 5.—Ability of various thymosin preparations to induce Lyt phenotype conversions. Cells from layer B of BSA gradient-fractionated bone marrow cells were incubated at 2×10^6 cells/ml in the indicated concentration of the various thymosin preparations for 90 mins at 37°C and analyzed for Lyt⁺ cells by microcytotoxicity assays.

TABLE 2.—Induction of helper T cells by incubation of bone marrow cells from T × BM mice in vitro with 100 μg/ml of thymosin fr. 5

C57BL/6 T × BM spleen cells cultured with—*	Pretreatment with thymosin fr. 5	Mean D-PFC/10^6 cells	
		Media	SRBC
—	–	18 ± 7	57 ± 16
Layer A	–	22 ± 9	36 ± 12
Layer A	+	26 ± 11	258 ± 47
Layer B	–	35 ± 16	69 ± 21
Layer B	+	28 ± 12	465 ± 67
Layer C	–	46 ± 9	75 ± 27
Layer C	+	32 ± 10	68 ± 31
Layer D	–	37 ± 17	59 ± 20
Layer D	+	49 ± 18	92 ± 18

*Spleen cells (7.5×10^6) were cultured in triplicate with 2.5×10^6 bone marrow cells from syngeneic mice which had been isolated on BSA gradients into layer A, B, C, or D and incubated with either calf spleen extract or thymosin fr. 5 for 90 mins at 37°C and washed prior to use. Cultures were incubated for 5 days and subsequently harvested and assayed for direct plaque-forming cells (D-PFC) against SRBC.

TABLE 3.—Functional analysis of thymosin α7-induced, BSA gradient-purified bone marrow cells in the anti-SRBC response in vitro*

T × BM spleen cells cultured with—	Addition of normal syngeneic helper cells	Mean ± SE D-PFC/10⁶ cells: in vitro response to—	
		Media	SRBC
—	–	15 ± 7	48 ± 16
Layer A	–	26 ± 12	15 ± 7
Layer B	–	32 ± 16	38 ± 20
Layer C	–	28 ± 10	24 ± 14
Layer D	–	34 ± 18	30 ± 16
—	+	38 ± 12	356 ± 54
Layer A	+	27 ± 8	210 ± 35
Layer B	+	20 ± 6	60 ± 18
Layer C	+	45 ± 10	292 ± 87
Layer D	+	36 ± 12	328 ± 45

*Spleen cells (7.5×10^6) from thymectomized, irradiated anti-thy + C′-treated syngeneic bone marrow cell-reconstituted mice were cultured in vitro in the absence or presence of normal syngeneic splenic T cells (2.5×10^6), along with bone marrow cells from 1×10^6 BSA gradient-purified fractions incubated for 90 mins with 100 μg/ml of thymosin fr. α7.

TABLE 4.—Lyt phenotype of the suppressor cell induced by thymosin α7

C57BL/6 T × BM spleen cells cultured with—	Pretreatment of the BSA gradient-fractionated cells*	Source of helper cells	Mean ± SE D-PFC/10⁶ cells	
			Media	SRBC
—	—	–	26 ± 10	101 ± 39
—	—	+	28 ± 12	437 ± 56
Layer B	—	+	41 ± 20	88 ± 34 †
Layer B (C57BL/6)	αLy 1.1 + C′	+	12 ± 5	26 ± 9
Layer B (C57BL/6)	αLy 2.1 + C′	+	32 ± 9	34 ± 15
Layer B (C57BL/6)	αLy 3.1 + C′	+	29 ± 7	29 ± 17
Layer B (C57BL/6)	αLy 1.2 + C′	+	38 ± 10	286 ± 48
Layer B (C57BL/6)	αLy 2.2 + C′	+	27 ± 12	327 ± 36
Layer B (C57BL/6)	αLy 3.2 + C′	+	46 ± 16	354 ± 57
Layer B (C57BL/6-Ly 1^a)	αLy 1.1 + C′	+	27 ± 9	387 ± 53 †
Layer B (C57BL/6-Ly 1^a)	αLy 2.1 + C′	+	18 ± 5	46 ± 11
Layer B (C57BL/6-Ly 1^a)	αLy 3.1 + C′	+	15 ± 6	53 ± 9
Layer B (C57BL/6-Ly 2^a3^a)	αLy 1.1 + C′	+	31 ± 14	61 ± 16
Layer B (C57BL/6-Ly 2^a3^a)	αLy 2.1 + C′	+	26 ± 11	394 ± 55 †
Layer B (C57BL/6-Ly 2^a3^a)	αLy 3.1 + C′	+	24 ± 7	218 ± 16) †

*Bone marrow cells from layer B of BSA gradients were induced with 100 ng/ml of thymosin fr. α7. Cultures were set up as described in the Materials and Methods section.

†$P < 0.05$.

2^+, and 3^+ by Cantor et al (15), recent results by Mosier et al (16) indicate that suppressor cells from neonatal mice were Lyt 1^+, 2^+, and 3^+, the same phenotype as the α7-induced suppressor cells.

Therefore, one possible level of control of T-cell maturation and subsequent T-cell function is the regulation of synthesis of the component polypeptides of thymosin.

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Control of Differentiation of Thymocyte Precursors in the Bone Marrow by Thymic Hormones [1]

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SUMMARY

Terminal deoxynucleotidyl transferase (TdT) is uniquely associated with the major thymocyte population and a minor bone marrow population which is separable on bovine serum albumin gradients. The expression of TdT in the bone marrow subpopulation is under thymic regulation in that this population is significantly reduced in nude mice or is lost with time after thymectomy. The expression of TdT in athymic mice can be induced both in vivo and in vitro by thymosin fraction 5 or by β_3, a peptide purified from thymosin fraction 5. The optimal concentration of β_3 in the in vitro induction of TdT is approximately 10 ng/ml. These results demonstrate that thymic hormones control the early differentiation of prothymocytes in the bone marrow.

[Cancer Treat Rep 62:1749–1755, 1978]

Although considerable research has dealt with the differentiation of immunologically mature T cells, few studies have addressed the control of differentiation of early T cells. The progression of T-cell differentiation is thought to begin in the bone marrow. Further maturation then occurs in the thymus and terminally differentiated T cells localize in peripheral lymphoid tissues. This sequence of differentiation has been indicated primarily by ablation and reconstitution types of experiments, since specific markers for early thymocytes have been difficult to identify. During the last few years, however, several studies have indicated that the enzyme terminal deoxynucleotidyl transferase (TdT) is uniquely associated with early T-cell differentiation (1–4). In vitro, TdT polymerizes deoxynucleotides, and although it requires a primer, it does not require a template (5). The function of the enzyme in vivo, however, is not known. TdT is found in the cortisone-sensitive, major thymus population, but not in immunologically committed, more mature T cells. Low levels of TdT are also found in bone marrow cells and this activity has been shown to be localized in a minor cell population separable by bovine serum albumin (BSA) gradient fractionation (6). This population of prothymocytes is Thy-1-negative, but can be induced in vitro to express Thy-1 by thymic hormones (7). In previous studies (8) and in the results presented here, we have studied the factors that control the differentiation of this prothymocyte population. The results demonstrate that a thymic hormone, β_3, induces TdT in an early prothymocyte population in bone marrow from athymic mice, demonstrating the thymic regulation of early prothymocyte differentiation in the bone marrow.

MATERIALS AND METHODS

Mice.—NIH Swiss nu/+, nu/nu, and C57BL/6 specific pathogen-free mice used in these experiments were obtained from the Frederick Cancer Research Center's Animal Production Area, Md. The mice were 6 weeks old at the beginning of the experiments.

Cell fractionation.—The method of Raidt et al (9) was used to fractionate different cell suspensions from spleen, thymus, lymph nodes, or bone marrow. Four layers of cells were formed at the interfaces of

[1] Supported by contract N01-CO-75380 from the Office of the Director, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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the discontinuous BSA gradient: fraction A between 10% and 23% BSA; fraction B between 23% and 26%; fraction C between 23% and 29%; and fraction D between 29% and 33%. BSA (Path-O-Cyte 5, lot Nos. 25 and 26) was obtained from Miles Research Products (Elkhart, Ind). Fractionated cells were washed three times in Ham's F-12 medium.

Enzyme preparation.—The procedure for enzyme extraction has been described previously (6).

TdT assay.—The TdT assay was adapted from Kung et al (3). One unit of enzyme activity was defined as the amount catalyzing the incorporation of 1 pmol of dGTP into acid-insoluble material per hour. The specific activity was calculated from the total enzyme activity recovered from phosphocellulose per 10^8 nucleated viable cells.

Anti-Thy-1 serum.—Rabbit anti-Thy-1 serum was prepared by injecting 1×10^7 thymocytes from $B6C3F_1$ mice three times at weekly intervals. The rabbit was bled 2 weeks after the last injection. The serum was decomplemented and absorbed on $B6C3F_1$ liver cells twice and on NIH Swiss nu/nu spleen cells three times.

Immunofluorescence.—The indirect immunofluorescence method of Cerottini and Brunner (10) was used to determine the frequency of Thy-1-positive cells. BSA-fractionated bone marrow cells, which had or had not been incubated with thymosin (50 ng/ml) for 2 hours at 37°C, were incubated with rabbit anti-Thy-1 serum (1:200) at 37°C for 30 minutes. The cells were centrifuged and washed three times in cold Ham's F-12 medium. The washed cells were suspended in 0.10 ml of a fivefold dilution of fluorescein-conjugated goat anti-rabbit IgG (Meloy Laboratories, Springfield, Va) and incubated at room temperature for 30 minutes. The cells were washed twice with Ham's F-12 medium and resuspended in a drop of Bacto FA mounting fluid, pH 7.2 (Difco Laboratories, Detroit, Mich). Smears of cells were made on the surface of microscope slides and were then examined by fluorescence microscopy.

Thymosin treatment in vivo.—Thymosin fraction 5 (lot No. BPM390) and spleen fraction 5 (lot No. 307), purified as previously described (11), were resuspended in saline solution at a concentration of 500 μg/ml. Ten daily injections of 100 μg were given ip to 6-week-old NIH Swiss nu/nu mice, which were killed 24 hours after the last injection. For the in vitro induction experiments, cells were washed three times after BSA gradient fractionation in Ham's F-12 medium containing 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10 μg/ml of gentamicin. After being washed, cell concentrations were adjusted to 1×10^8 cells/dish in 20 ml of Ham's F-12 medium containing antibiotics, 5% fetal bovine serum (Flow Laboratories, Rockville, Md), and different concentrations of thymosin or spleen fraction 5 or of any of the different peptides isolated from thymosin. After incubation for various periods of time, cells were collected, washed twice with Ham's F-12 medium, and extracted as previously described for TdT activity (6). The following peptides, isolated from thymosin, were assayed for their ability to induce TdT in vitro: α_1, α_5, α_7, β_1, β_3, β_4, and synthetic α-1.

Actinomycin D treatment and [^{3}H]uridine labeling.—Actinomycin D (0.5 μg/ml) was incubated for 12 hours in the presence of thymosin with fraction B from the bone marrow of NIH Swiss nu/nu mice to study its effect on TdT induction. To follow the effect of actinomycin D on RNA synthesis, cells were labeled with [^{3}H]uridine (10 microcuries/ml) for the last 90 minutes of incubation.

RESULTS

TdT has been shown to be detectable only in the thymus and bone marrow (2–4). In order to better assess the distribution of TdT in these tissues, we examined thymus and bone marrow cell populations from NIH Swiss mice fractionated on discontinuous BSA gradients for TdT activity. As illustrated in table 1, TdT activity is evenly distributed in fractions A, B, and C of fractionated thymocytes. These fractions contain 80% of the initial thymocytes. Using NIH Swiss thymocytes, only the peak I TdT activity was detectable, which is consistent with previous data demonstrating an age- and strain-dependent difference in peak II TdT expression (12). In contrast to thymocytes, however, fractionated bone marrow cells from NIH Swiss mice primarily had TdT activity associated with fraction A. Although this fraction constitutes only 5% of the total bone, the specific activity of TdT in these cells was comparable to thymocytes, suggesting that the majority of the cells in fraction A are TdT-positive. Also shown in table 1 are the results obtained with bone marrow from NIH Swiss nu/nu mice. In contrast to the thymic-bearing NIH Swiss mice, only approximately 10% of the TdT activity was detectable. Comparable results (not shown) have been obtained with C57BL/6 mice in that TdT is found in fractions A, B, and C of thymus cells, while only fraction A of bone marrow cells expresses TdT. Similarly, thymectomy of 4-week-old C57BL/6 mice is followed by a rapid decrease of TdT-positive cells in the bone marrow. These results demonstrate that TdT is associated with most thymocyte subpopulations and a minor bone marrow population, presumably a prothymocyte population, and that the latter population may be under thymic regulation.

TABLE 1.—Distribution of TdT activity in fractionated tissues from NIH Swiss nu/+ and nu/nu mice

	BSA fractions							
	A		B		C		D	
Tissue	Peak I	Peak II	Peak I	Peak II	Peak I	Peak II	Peak I	Peak II
Thymus, NIH nu/+	185*	7	193	3	161	< 0.1	< 0.1	< 0.1
Bone marrow, NIH nu/+	145	< 0.1	6	< 0.1	1	< 0.1	< 0.1	< 0.1
Bone marrow, NIH nu/nu	16	3	< 0.1	1	1	< 0.1	< 0.1	< 0.1

*pmols of dGTP incorporated/hr/10^6 cells.

TABLE 2.—In vivo induction of TdT activity in bone marrow cells from nude mice with thymosin fraction 5*

	BSA fractions							
	A		B		C		D	
Treatment	Peak I	Peak II	Peak I	Peak II	Peak I	Peak II	Peak I	Peak II
Saline	22†	4	9	1	< 0.1	< 0.1	< 0.1	< 0.1
Spleen fraction 5	18	< 0.1	10	< 0.1	3	< 0.1	< 0.1	< 0.1
Thymosin fraction 5	210	3	32	< 0.1	3	< 0.1	2	< 0.1

*NIH Swiss nu/nu mice were given 10 daily injections ip of spleen fraction 5, thymosin fraction 5, or saline. 24 hrs after the last injection, the mice were killed and TdT was isolated as described in the Materials and Methods section.

†pmols of dGTP incorporated/hr/10^6 cells.

The restoration of various T-cell fractions and the regulation of T-cell differentiation of bone marrow cells have been shown to be influenced by thymic hormones (13–15). One such effect has been the ability of thymosin fraction 5 to induce the expression of theta in vivo (11). We therefore examined BSA gradient fractions of bone marrow cells for the expression of theta and for their inducibility for theta expression. As shown in table 2, the majority of bone marrow cells are theta-negative by immunofluorescence, including fraction A which is TdT-positive. These results demonstrate that TdT and theta antigen expression are independent and that TdT expression may precede theta expression in T-cell differentiation. However, if fractionated bone marrow cells are incubated in vitro with thymosin fraction 5, fraction A is inducible for theta expression. These results are consistent with previous studies suggesting that thymic hormones induce theta expression in TdT-positive bone marrow cells (7).

In order to next examine the effect of thymosin fraction 5 on TdT expression, we treated NIH Swiss nu/nu mice with thymosin fraction 5 and assayed fractionated bone marrow cells for TdT activity. As shown in table 3, ten daily injections of 100 μg of thymosin fraction 5 increased TdT activity in the bone marrow fraction A to levels comparable to their heterozygous littermates. The induction is specific for thymosin fraction 5 in that neither saline nor spleen fraction 5 treatment had any effect. Interestingly, thymosin treatment of NIH Swiss nu/nu mice resulted in the appearance of peak I activity; however, when the same treatment is given to thymectomized C57BL/6 mice, both peaks I and II were induced to a specific activity comparable to that of the normal controls (data not shown). No TdT activity was detected in fractionated cells from either the spleen or the lymph nodes of thymosin-treated mice (data not shown). The results obtained after in vivo treatment with thymosin suggested that this hormone could promote TdT expression in bone marrow. However, it was not possible to differentiate between a direct inductive effect and a secondary effect on differentiation. We therefore examined the ability of thymosin fraction 5 to induce TdT in vitro in fractionated bone marrow and spleen cells from NIH Swiss nu/nu mice. The results are shown in figure 1. When fractionated spleen cells were treated with 25 ng/ml of thymosin fraction 5 for 18 hours, TdT was specifically induced in fraction B of bone marrow cells. Again, only peak I activity was induced. This time, however, the specific activity obtained was about 60% of the normal bone marrow fraction A population. Spleen fraction 5 had no inductive effect in

TABLE 3.—Expression of Thy-1 in fractionated bone marrow cells from C57BL/6 mice before and after in vitro incubation with thymosin fraction 5

	% fluorescent cells*			
	BSA fractions			
	A	B	C	D
Bone marrow before thymosin	3.5	2.5	1.3	2.5
Bone marrow after thymosin	66.6	11.3	1.5	3.0

*Thy-1-positive cells were determined by indirect immunofluorescence as described in the Materials and Methods section.

vitro. These results, therefore, suggest a direct role of thymosin fraction 5 in the induction of TdT activity in the bone marrow cells from nu/nu mice.

The kinetics of induction using 25 ng/ml are shown in figure 2. The induction in vitro is rapid so that within 2 hours a significant increase in TdT is evident. By 4–6 hours, the cells are fully induced, and the enzyme activity remains constant up to 24 hours. Also shown in figure 2 are the results obtained at 12 hours in the presence of 0.5 μg/ml of actinomycin D. Under these conditions, > 95% of the RNA synthesis is inhibited (data not shown), as is the induction of TdT.

Since thymosin fraction 5 is a mixture of several different peptides, we have studied the ability of several purified peptides to induce TdT in vitro in BSA fraction B from the bone marrow. As shown in table 4, most of the peptides did not induce TdT. However, in the β group, β_4 was able to induce TdT to 30% of the thymosin fraction 5 level and β_3 had the highest activity and induced TdT to values close to 80% of that obtained with thymosin fraction 5. Structural analysis of β_3 and β_4 has shown that these two single peptides are closely related in structure (unpublished results). Nevertheless, the results demonstrate that purified thymic hormones can induce TdT expression.

The concentration dependence for the induction of TdT in bone marrow fraction B cells by thymosin fraction 5, α_1, and β_3 is shown in figure 3. For

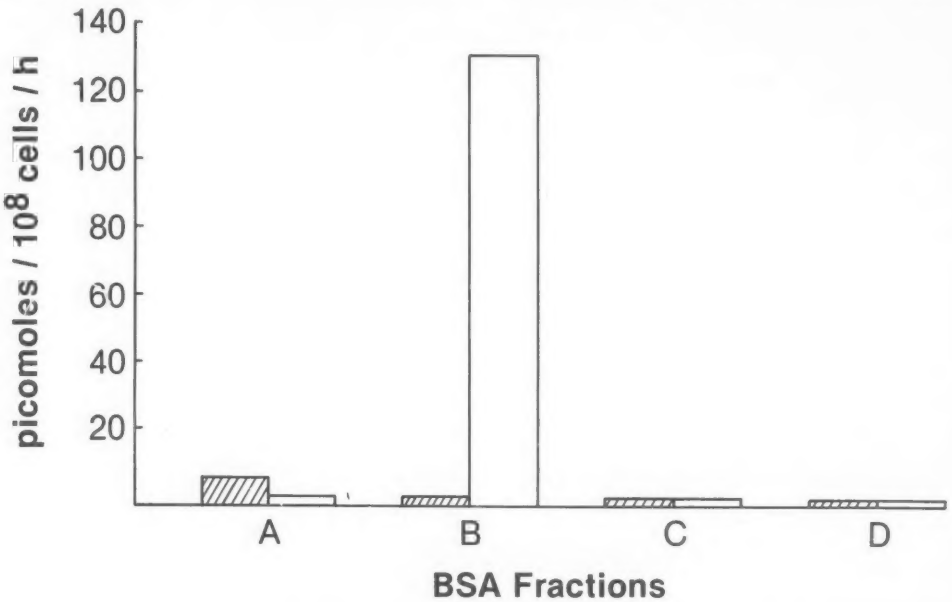

FIGURE 1.—In vitro induction of TdT in fractionated spleen and bone marrow cells: bone marrow and spleen cells from NIH Swiss nu/nu mice were fractionated on BSA gradients and incubated for 18 hrs with 75 ng/ml of thymosin fraction 5. The cells were subsequently extracted and TdT activity was determined following phosphocellulose chromatography. Open bars = bone marrow cells; notched bars = spleen cells.

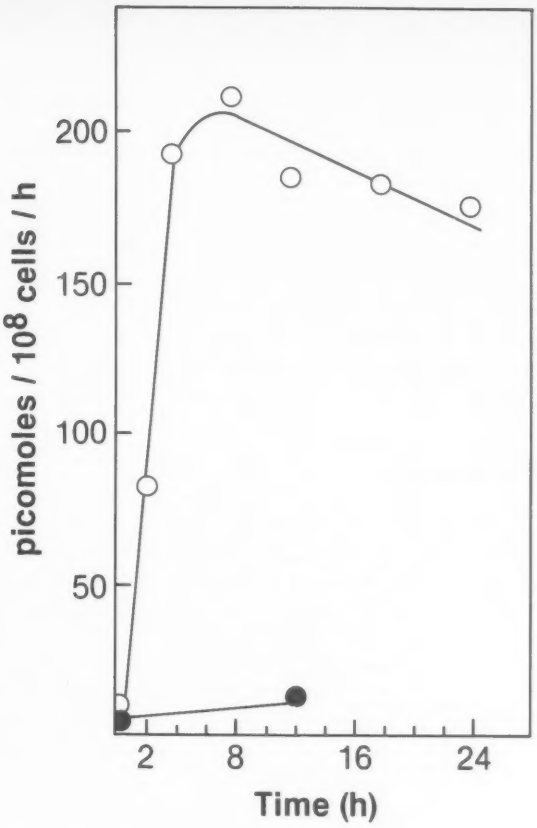

FIGURE 2.—Time dependence for the induction of TdT by thymosin fraction 5: NIH Swiss nu/nu bone marrow cells were fractionated on BSA gradients and the B layer was used. The cells were treated with 25 ng/ml of thymosin fraction 5 in the presence (●——●) or absence (○——○) of 0.5 μg/ml of actinomycin D. At the indicated times, aliquots of the cells were collected and extracted, and TdT activity was determined following phosphocellulose chromatography.

thymosin fraction 5, concentrations as low as 3 ng/ml had a significant effect and approximately 30 ng/ml was optimal; however, at higher concentrations, there was a significant inhibitory effect, which was not associated with a loss of cell viability. In contrast, α_1 did not show any induction at concentrations ranging from 2 to 200 ng/ml. β_3 showed significant TdT induction at 1 ng/ml and the optimum was achieved at 10 ng/ml. In contrast to thymosin, however, no inhibition of induction was observed even at concentrations of 500 ng/ml. These results suggest that thymosin fraction 5 and β_3 specifically induce TdT in a manner comparable to the induction observed for other enzymes in response to specific hormones.

TABLE 4.—In vitro induction of TdT in fraction B bone marrow cells from NIH Swiss nu/nu mice

Treatment	TdT specific activity*
Control saline	2.0
Spleen fraction 5	1.0
Thymosin fraction 5	214.0
α-1	5.2
α-5	< 0.1
α-7	< 0.1
β1	2.5
β3	145.0
β4	58.0
Synthetic α-1	3.0

*Values for peak I activity only (pmols of ^{3}H-dGTP/hr/10^8 cells). Incubation was for 18 hrs with 50 ng/ml of each peptide.

DISCUSSION

Thymic hormones have been implicated in the regulation of differentiation of immune cells (13–15). Most of these studies have measured increases in immunologic parameters such as mitogen responses, MLR activity, or changes in serologically defined cell surface antigens. Therefore, the influence of thymic hormones on the early stages of thymocyte differentiation has not been assessed. In the studies presented here, we have directly addressed this question by studying thymic hormone

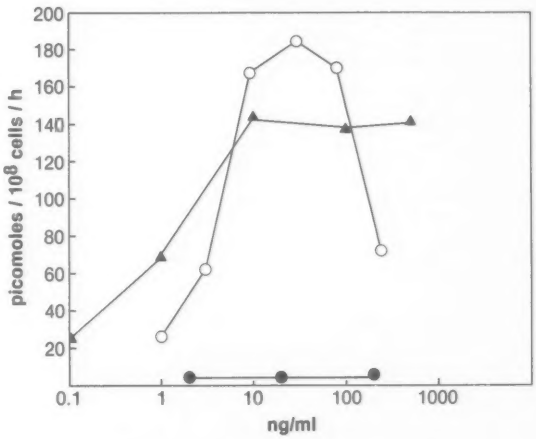

FIGURE 3.—Concentration dependence for the induction of TdT by thymic hormones: NIH Swiss nu/nu bone marrow cells were fractionated on BSA gradients and the B layer was used for induction of TdT. The cells were treated with varying concentrations of thymosin fraction 5 (○——○), α-1 (●——●), or β_3 (▲——▲), for 12 hrs, and the cells were extracted. TdT activity was determined following phosphocellulose chromatography.

regulation of TdT in early prothymocyte populations. The results demonstrate that TdT is an excellent marker enzyme for what is thought to represent the early stages of thymocyte differentiation. In particular, TdT in the thymus has been shown to be associated with the cortisone-sensitive, major thymocyte population and is absent in the more immunologically mature cortisone-resistant populations (3,4). Similarly, TdT is not associated with mature, peripheral T cells in the spleen or lymph nodes. As previously demonstrated (6) and shown here, TdT is also found in a minor bone marrow subpopulation. This cell population is phenotypically TdT-positive and Thy-1-negative. Silverstone et al (7) have suggested that this population is a prothymocyte population based on the observation that thymic hormones can induce Thy-1 expression in these cells. This observation has been confirmed in our studies. In addition to this cell population, we have described another cell population which phenotypically is Thy-1-negative and TdT-negative, but which characteristically is inducible by thymosin fraction 5 or specifically β_3 to express TdT. This cell presumably represents one of the earliest precursors in thymocyte differentiation.

Our results demonstrate that the thymosin fraction 5 responsive bone marrow cells can be readily demonstrated in fraction B of BSA gradient fractionated bone marrow cells from nude or thymectomized mice in in vitro induction experiments. These results demonstrate that the emergence of this stem cell population is thymus-independent, but that its continued differentiation in the bone marrow requires thymic hormones. Interestingly, in normal thymus-bearing mice, fraction B of bone marrow cells is not inducible for TdT (unpublished observation), suggesting that in such mice there is sufficient hormone to immediately promote differentiation. Thus, the limiting event in early thymocyte differentiation is the thymic independent generation of precursor cells in the bone marrow. Consequently, only in athymic mice does this precursor cell accumulate. Whether such cells are obligatorily committed at this point to thymocyte differentiation or can undergo differentiation to other lymphoid components is an interesting but unanswered question.

The concentration-dependent curves for thymosin fraction 5 and β_3 induction of TdT showed significant differences. In particular, the bell-shaped induction curve for thymosin fraction 5 illustrated a significant inhibition of TdT induction at high concentrations without a loss in cell viability. In contrast, β_3 induction showed no such inhibition at high concentrations. These results suggest that other hormones are present in thymosin fraction 5 which either directly inhibit the ability of the cells to respond or induce differentiation along alternative pathways. These questions are currently being examined by identifying specific peptides that inhibit the ability of bone marrow cells to respond to β_3.

In summary, our data demonstrate that thymic hormones and specifically β_3 are obligatory for the differentiation of early, bone marrow localized thymocytes. The biologic properties of this response suggest that β_3 induction of TdT is very similar, if not identical, to the induction of a variety of differentiation specific enzymes by hormones. The kinetics and concentration dependence for this induction are compatible with a physiologically significant role for this response in vivo.

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Characterization of Thymosin-Responsive Peripheral Blood Null Cells [1]

Joseph Kaplan [2,3,*]

SUMMARY

Suspensions of human peripheral blood lymphocytes were enriched for E rosette-negative (E^-) surface immunoglobulin-negative (SIg^-) "null" cells expressing either human T or human B lymphocyte antigens. These enriched suspensions were then tested for responsiveness to activation of E rosette formation by thymosin. Thymosin activated some human T lymphocyte antigen-positive E^-SIg^- but not human B lymphocyte antigen-positive E^-SIg^- cells to form E rosettes. Therefore, thymosin-responsive peripheral blood null cells appear to be precommitted to the T-cell line of differentiation.

[Cancer Treat Rep 62:1757–1761, 1978]

Human peripheral blood T lymphocytes can be identified by their ability to form rosettes with sheep erythrocytes, and B lymphocytes can be identified by detection of surface immunoglobulin (SIg') (1,2). Approximately 10%–20% of blood lymphocytes possess neither of these two phenotypic markers, ie, they are E rosette-negative (E^-) and SIg-negative (SIg^-), and have been termed null cells (3–5). The nature of these cells and their relation to T and B lymphocytes have not been well-defined. Using reciprocally absorbed rabbit antisera to autologous human T and B lymphoblast cell lines, we have found (6,7) that most, if not all, peripheral blood null cells express either human T lymphocyte antigens (HTLA) or human B lymphocyte antigens (HBLA). These findings suggest that some null cells may be related to the T-cell lineage and others to the B-cell lineage.

Previous studies have shown that thymosin and other purified thymic extracts activate the ability of some peripheral blood and bone marrow null cells to develop the capacity of mature T cells to bind sheep erythrocytes (8,9). It has also been shown that the number of thymosin-inducible cells in the blood is proportional to the number of circulating null cells (10,11) and that the increases in circulating E rosette-positive (E^+) cells which occur after in vivo administration of thymosin are accompanied by proportional decreases in circulating null cells (12,13). Taken together, these findings have suggested that the null cell population found in peripheral blood contains immature T-cell precursors which can be induced by thymic hormones to further differentiate into E^+ cells. In the studies presented here, we demonstrate that of the two major types of peripheral blood null cells, $HTLA^+$ and $HBLA^+$ null cells, only those which express HTLA respond to thymosin activation of E rosette formation. The results imply that thymosin-responsive peripheral blood null cells are precommitted to the T-cell line of differentiation.

MATERIALS AND METHODS

Preparation of rabbit anti-HTLA and anti-HBLA sera.—The preparation and specificity testing of the anti-HTLA and anti-HBLA heteroantisera have been previously described in detail (6,7). Briefly, rabbits were immunized with either T-lymphoblast cell line HSB-2 or the autologous B-lymphoblast cell line SB (14,15). The resulting antisera were then reciprocally absorbed with the opposite cell line until they were no longer cytotoxic in the presence of complement for the absorbing cell type.

Source and preparation of lymphocytes.—Periph-

[1]Supported by Public Health Service grants CA-17534 and CA-00188 and contract 1-CP-33333 (Division of Cancer Cause and Prevention) from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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[3]I thank Teresa Sun, Cheryl Fox, and Gabe Haas for excellent technical assistance.

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eral blood lymphocytes were prepared by Ficoll-Hypaque gradient separation of leukocytes obtained as a byproduct of plateletpheresis from normal adult donors and from children with sickle cell anemia undergoing prophylactic partial exchange transfusion.

Null cell preparation.—Null cell-enriched suspensions were prepared by sequential nylon column filtration and E rosette depletion of peripheral blood lymphocytes as previously described (16). Such suspensions were then further enriched for either $HTLA^+$ or $HBLA^+$ null cells by treatment with either anti-HBLA plus complement or anti-HTLA plus complement; suspensions containing 10^7 cells/ml of diluent (RPMI-1640) with 20% fetal calf serum and 10 μg/ml of gentamycin were incubated with an equal volume of appropriately diluted rabbit serum as a source of complement. After 60 minutes of incubation at 37°C, the cells were washed three times in diluent prior to further testing.

Detection of cell surface markers.—Cells were tested for E rosette formation with neuraminidase-treated sheep erythrocytes (17) and for SIg by methods previously described (18). The cell surface expression of HTLA and HBLA was assayed by indirect immunofluorescence using rabbit anti-HTLA and anti-HBLA and fluorescein-conjugated goat anti-rabbit IgG (Hyland Labs, Los Angeles, Calif) (6) or by complement-dependent cytotoxicity (19).

Assay of thymosin responsiveness.—Null cells were tested for their ability to respond to thymosin activation of E rosette formation using a method similar to that previously described by Wara et al (13). Lymphocytes, 2.5×10^5 cells in 0.05 ml of RPMI-1640, were mixed with 0.05 ml of thymosin, 0.1 mg/ml (kindly supplied by Dr. A. Goldstein). Control cells were mixed with 0.05 ml of RPMI-1640. After 10 minutes of incubation at 37°C, a 0.1-volume of 2% neuraminidase-treated sheep erythrocytes in Hanks' balanced salt solution was added to each tube. The cell suspensions were then incubated for 15 minutes at 37°C, centrifuged at 200 $\times$ *g* for 5 minutes, and then further incubated for 2–18 hours at 4°C. The proportion of E rosettes in each mixture was then determined by examining duplicate wet cell preparations. The difference in the percent of E rosettes between mixtures containing thymosin and mixtures containing diluent alone were taken as an index of the percent of thymosin-responsive cells in the original suspensions.

RESULTS

Peripheral blood lymphocytes obtained from seven children with sickle cell anemia undergoing monthly prophylactic partial exchange transfusion (subjects 1–7) and one healthy adult donor undergoing plateletpheresis (subject 8) were depleted of SIg^+ B cells by nylon column filtration and of E^+ T cells by E rosette depletion on Ficoll-Hypaque gradients. Cell surface marker determinations on the remaining cells (table 1) showed that (*a*) between 73% and 93% were E^-SIg^- null cells, (*b*) considerably more cells were $HTLA^+$ and $HBLA^+$ than were E^+ and SIg^+, (*c*) the proportions of $HTLA^+$ cells were inversely related to the proportions of $HBLA^+$ cells, and (*d*) the sums of $HTLA^+$ and $HBLA^+$ cells in most cases approximated 100%. As previously discussed (6,7), these findings indicated that E^-SIg^- null cells express HTLA and HBLA, and that some null cells express HTLA whereas others express HBLA. This was confirmed by experiments in which null cell suspensions were treated with

TABLE 1.—T- and B-lymphocyte markers in null cell-enriched suspensions*

	% positive cells			
Subject	E rosette	SI_$	HTLA	HBLA
1	18	9	46	56
2	8	7	55	49
3	12	2	NT†	NT
4	16	3	61	31
5	6	1	NT	NT
6	4	4	NT	NT
7	4	4	NT	NT
8	18	3	71	14
Mean ± SD	9 ± 6	4 ± 3	59 ± 14	39 ± 16

*Null cell-enriched suspensions were prepared by sequential nylon fiber column filtration and E rosette depletion of peripheral blood mononuclear cells.
†NT = not tested.

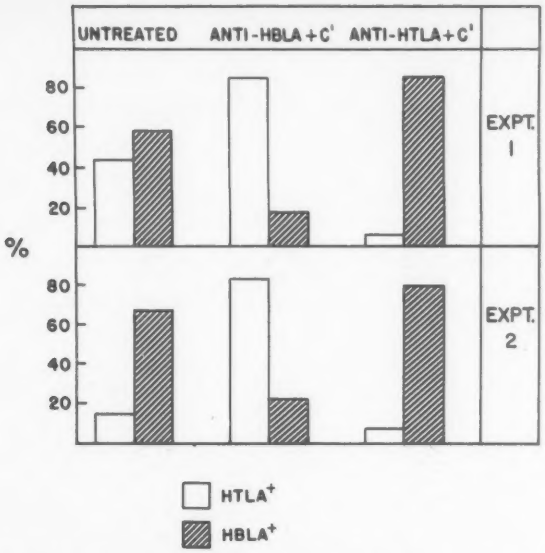

FIGURE 1.—Proportions of HTLA+ and HBLA+ cells in null cell-enriched lymphocyte suspensions before and after treatment with anti-HBLA or anti-HTLA and complement as determined by complement-dependent cytotoxicity.

anti-HTLA or anti-HBLA plus complement and then tested for reactivity with each antiserum after removing dead cells by Ficoll-Hypaque gradient separation. As shown in two representative experiments (fig 1), compared to untreated cells, null cell-enriched suspensions treated with anti-HBLA plus complement were depleted of HBLA+ cells and enriched in HTLA+ cells, whereas suspensions treated with anti-HTLA plus complement were depleted of HTLA+ cells and enriched in HBLA+ cells.

In addition to demonstrating that the HTLA+ and HBLA+ null cell subsets were, by and large, distinct populations, these experiments also demonstrated the feasibility of preparing suspensions specifically enriched in HTLA+ or HBLA+ null cells. Using such suspensions, we proceeded to compare the T-cell precursor activity of HTLA+ and HBLA+ null cells as measured in vitro by thymosin activation of E rosette formation (13). Data from a representative experiment are given in table 2, and the combined results of eight separate experiments are shown graphically in figure 2. Thymosin induced an increase in the percent of E+ cells in intact null cell suspensions, and induced an even greater increase in the percent of E+ cells in null cell suspensions pretreated with anti-HBLA plus complement. By contrast, null cell suspensions pretreated with anti-HTLA plus complement showed little or no increase

TABLE 2.—Thymosin activation of E rosette formation in null cell-enriched suspensions before and after depletion of HBLA+ or HTLA+ cells

Treatment	% E rosettes (diluent)	% E rosettes (thymosin)	Change in % E rosettes*
None	4	11	+ 7
Anti-HBLA + C'	8	26	+ 18
Anti-HTLA + C'	4	4	0

*Change in % E rosettes = % E (thymosin) – % E (diluent).

in the percent of E+ cells when incubated with thymosin.

These findings implied that thymosin induced HTLA+ but not HBLA+ null cells to become E+. An alternative possibility considered was that thymosin caused a selective loss of E− cells resulting in a relative rather than an absolute increase in E+ cells. Since thymosin induced an increase in the percent of E+ cells only in suspensions containing HTLA+ cells, such a selective loss would have had to be of HTLA+E− cells rather than HBLA+E− cells resulting in a decrease in the percent of HTLA+ cells. However, when tested by complement-dependent cytotoxicity, the percent of HTLA+ cells in null cell suspensions did not decrease after incubation with sheep erythrocytes and thymosin. Therefore, the increase in the percent of E+ cells in thymosin-

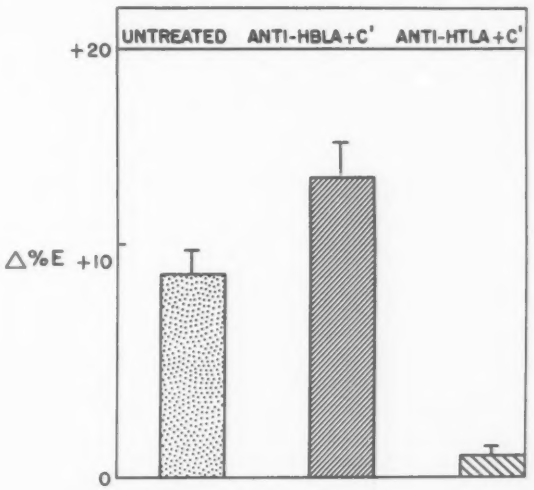

FIGURE 2.—Responsiveness to thymosin activation of E rosette formation by null cell-enriched lymphocyte suspensions before and after treatment with anti-HBLA and anti-HTLA and complement. The change in % E rosettes was calculated as follows: % E rosettes (thymosin)-% E rosettes (diluent). Values presented = mean ± SEM of 8 separate experiments.

treated null cell suspensions most likely resulted from in vitro differentiation of $HTLA^+E^-$ cells into $HTLA^+E^+$ cells.

DISCUSSION

The observation that thymosin-responsive null cells express the same HTLA as mature T lymphocytes implies that such cells are precommitted to the T-cell line of differentiation. This would be consistent with evidence in mice that thymic hormone-inducible cells are already programmed to become T cells (20). Studies in mice also suggest that there are at least two different types of committed T-cell precursors: pre-thymic "T_0" cells which can be specifically induced by thymic hormones in vitro to express the murine T-cell antigens Thy 1 and TL (20), and postthymic immunoincompetent "T_1" cells which already express Thy 1 antigens and are induced in vitro by thymic hormones to become functionally mature "T_2" cells (21,22). Although activation of E rosette formation does not necessarily correspond to activation of T-cell immunocompetence, if human T-cell differentiation parallels this scheme of mouse T-cell differentiation, the fact that human thymosin-responsive peripheral blood lymphocytes already express T-lymphocyte differentiation antigens would suggest that they are the equivalent of post-thymic "T_1" cells.

Although thymosin-inducible blood lymphocytes appear to be $HTLA^+$ null cells, consideration of the results in table 1 and figure 2 suggests that not all $HTLA^+$ null cells are thymosin-inducible. In fact, in these experiments an average of only 20% of the $HTLA^+$ null cells were activated by thymosin to form E rosettes. A relative insensitivity of the E rosette activation assay in measuring thymosin induction of T-cell maturation could explain this. However, no further increase in the percent of E^+ cells occurred when incubation time with thymosin was increased from 2 to 18 hours or when thymosin concentration was increased from 0.1 to 0.4 mg/ml. Therefore, the possibility must be considered that the peripheral blood $HTLA^+$ null cell population contains T-cell precursors at several different stages of maturation, and that some of these are more readily induced by thymic hormones than others. This receives indirect support from evidence that there are differences in mode of response and dose response to thymic hormones by T-cell precursors from blood and bone marrow (8,24).

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Suppressor Cell Activity in Cancer Patients: A Possible Role for Thymic Hormones [1]

Yehuda Z. Patt,* Evan M. Hersh, Karel Dicke, Gary Spitzer, and G. M. Mavligit [2,3]

SUMMARY

We have identified the presence of suppressor cell activity in the peripheral blood of immunosuppressed stage IV cancer patients. The patients' cells had a diminished mitogenic response to phytohemagglutinin (PHA) and suppressed the PHA mitogenic response of a normal donor's peripheral blood lymphocytes (PBL). Thus, PBL from a cancer patient whose PHA mitogenic response was 3932 counts per minute (cpm), cultured together with a normal donor's PBL with a PHA mitogenic response of 82,865 net cpm, caused a > 50% reduction in the latter (37,651 net cpm). Suppressor cell activity was present in 12 of 14 patients tested. This effect was partially mitigated by irradiation with 4000 rads in nine of 14 patients. Preincubation of the patients' cells with thymosin followed by the addition of thymosin to the co-cultured cells mitigated the suppressor activity in five of ten patients and thymic humoral factor did the same in four of 11 patients. A radiosensitive and thymic hormone-responsive suppressor cell may be present in the peripheral blood of cancer patients. Confirmation of this preliminary observation and further characterization of the cell or cells is currently being undertaken.

[Cancer Treat Rep 62:1763–1768, 1978]

Although the concept of immunosurveillance as a barrier against tumor development (1,2) is currently being challenged, the immune defect accompanying tumor growth is well established (3,4). A decreased phytohemagglutinin (PHA) mitogenic response was demonstrated in the human (4,5) and in the animal (6) tumor-bearing host. This immune suppression is related at least in part to the existence of a suppressor cell depressing the immune response to the tumor (7) and decreasing the response to other antigens (8–11).

A prostaglandin-producing glass adherent suppressor cell (12) as well as a nonphagocytic lymphocyte suppressor cell (13) have been demonstrated in Hodgkin's disease patients. Nonspecific suppressor cell activity sensitive to carrageenan and thus presumably monocytic in origin was also identified (14). A T-suppressor cell has been previously described (11,13). All of these suppressor cells are found in the peripheral blood of cancer patients. The preliminary data reported here describe a suppressor cell population in the peripheral blood of cancer patients which has the following characteristics: (*a*) the cells are partially sensitive to irradiation (4000 rads); and (*b*) the suppressor activity can be reduced in some of the patients by thymic hormones, ie, thymosin, and thymic humoral factor (THF) in some of the patients.

[1]Supported in part by Public Health Service grants CA-05831 and CA-14984 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, and by grants from Hoffmann-La Roche, Inc, and Miles Laboratories, Inc.

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[3]We are greatly indebted to Allan L. Goldstein, PhD (Division of Biochemistry, University of Texas Medical Branch, Galveston, Tex) and Nathan Trainin, MD (Department of Cell Biology, The Weizman Institute of Science, Rehovot, Israel) for their collaboration and supply of thymic hormones, and to Marvette Washington and Ruth Goldman for their excellent technical assistance.

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TABLE 1.—Patient characteristics and comparative effect of irradiation, thymosin, and THF on suppressor cell activity

Patient No.	Diagnosis	Prior treatment*	Presence of suppression	Mitigation by—		
				Radiotherapy	Thymosin	THF
1	Sarcoma	Surgery	+	+	–	–
2	Sarcoma	CyVADIC	–	–	–	–
3	Adenocarcinoma, lung	FACP	+	+	–	–
4	Adenocarcinoma, lung	Surgery	–	–	–	–
5	Adenocarcinoma, lung	—	+	+	ND†	–
6	Squamous cell carcinoma, lung	—	+	–	+	+
7	Adenocarcinoma, lung	Radiotherapy + FACP × 3	+	–	–	–
8	Oat cell carcinoma, lung	Radiotherapy + VIVA + CP	+	+	ND	ND
9	Oat cell carcinoma, lung	Radiotherapy + VIVA + CP	+	+	ND	ND
10	Embryonal carcinoma, testes	VB and DDP	+	+	+	+
11	Embryonal carcinoma, testes	VB and DDP	+	+	+	–
12	Embryonal carcinoma, testes	VB, DDP, actinomycin D, and BRM	+	+	ND	ND
13	Diffuse histiocytic lymphoma	CHOP and BMR	+	+	+	+
14	Carcinoma, colon	Radiotherapy	+	–	+	+

*CyVADIC = cyclophosphamide, vincristine, adriamycin, and DTIC; FACP = ftorafur, adriamycin, cyclophosphamide, and DDP; VIVA + CP = vincristine, isophosphamide, VP-16-213, and adriamycin + *C. parvum*; VB = vinblastine and bleomycin; CHOP = cyclophosphamide, adriamycin, vincristine, and prednisone.

†ND = not determined.

MATERIALS AND METHODS

Patient and Normal Donor Populations

The experiments were conducted on mononuclear cells isolated on a Ficoll-Hypaque gradient from the peripheral blood of 14 cancer patients with the diagnoses shown in table 1. Five patients with adenocarcinoma or squamous cell carcinoma of the lung were studied. Three patients had had no previous chemotherapy and two had been treated with a combination of ftorafur, adriamycin, cyclophosphamide, and *cis*-dichlorodiammineplatinum(II) (DDP), and iv hyperalimentation. Two patients had oat cell carcinoma of the lung and were previously treated with vincristine, isophosphamide, VP-16-213, and adriamycin in combination, with the addition of *Corynebacterium parvum* immunotherapy. Radiotherapy to the brain was used in one patient and radiotherapy to the chest in the other. Three patients with embryonal carcinoma of the testes had been previously exposed to vinblastine, bleomycin, and DDP. One patient had also been exposed to actinomycin D, and to autologous bone marrow reconstitution (BMR) following high doses of nitrosourea (BCNU), isophosphamide, and VP-16-213. Two patients with sarcoma were studied. One patient had failed to respond to a combination containing cyclophosphamide, vincristine, adriamycin, and DTIC and the other patient was tested before chemotherapy. A patient with diffuse histiocytic lymphoma failed to respond to treatment with cyclophosphamide, adriamycin, vincristine, and prednisone followed by VP-16-213. She was subsequently treated with high doses of BCNU, isophosphamide, and VP-16-213 followed by BMR. One patient with colon cancer was tested following radiotherapy to the primary lesion (table 1). All of the patients had stage IV disease. There were four women and ten men, ranging in age from 22 to 66 years.

Normal donors were hospital personnel ranging in age from 25 to 45 years. All subjects had previously shown a normal mitogenic response to PHA. Peripheral blood mononuclear cells were isolated from venous blood on a Ficoll-Hypaque gradient as previously described by Boyum (15); they were washed twice in RPMI-1640 and divided into aliquots.

Thymosin fraction V.—This is a thymic extract, isolated by Goldstein et al (16) and kindly supplied by Hoffmann-La Roche Co (Nutley, NJ), composed of at least 12–15 polypeptides ranging in molecular weight from 1000 to 15,000 daltons (16). This extract has been shown to induce pre-T as well as T_1

lymphocytes to undergo immunologic maturation. Its activity has been demonstrated in vitro and in vivo in nude and thymectomized (neonatal) mice and in patients with immunodeficiency diseases and cancer.

THF.—This was isolated and kindly supplied by Trainin et al (17). It is a polypeptide composed of 31 amino acids and has a molecular weight of 3220. The target of THF activity is a committed immature T_1 lymphocyte. Thus, THF has no activity in nude mice which are devoid of T_1 lymphocytes (17). Its activity is cAMP-mediated (18). Immunorestorative capacity has been demonstrated primarily in immunosuppressed patients (19) and in patients who have viral infections (20).

Exposure to thymic hormones.—A suspension containing 1×10^6 peripheral blood mononuclear cells/ml was incubated at 37°C for 1 hour in a shaking bath with either 100 μg/ml of thymosin fraction V or 100 μg/ml of THF or without any additive. In addition to this preincubation step, the thymic hormones have also been added to the cultured cells in the microtiter plate wells wherever indicated. Thymosin fraction V was added at a dose of 100 μg/well and THF at a dose of 20 μg/well.

Irradiation.—Cells were exposed to 4000 rads of gamma irradiation from a cesium source in tissue culture tubes containing a suspension of 10×10^6 cells/ml.

PHA mitogenic response.—This was measured by culturing the cells in microtiter plates as previously described (21). Lymphocytes (1.5×10^5) from either normal or patient peripheral blood were added to each well and then exposed to PHA–M (Difco Laboratories, Detroit, Mich) at a concentration of 20 μl of a 1:10 diluted solution per well for 72 hours. The cultures were pulsed with 1 microcurie of [^{3}H]-thymidine for the final 8 hours before cooling (4°C) and harvesting by a multiple sample harvester onto glass fiber filter for liquid scintillation counting (21). To demonstrate suppression, 1.5×10^5 normal cells were cultured together with 1.5×10^5 patient or normal cells in the same well, and the response to PHA was measured. The effect of irradiation on suppression was tested by culturing normal cells with patient or normal cells previously irradiated and then exposing the mixed populations to PHA. In order to test the effect of thymic hormones on suppression, 1.5×10^5 normal or patient cells previously treated with THF or thymosin were added to wells containing an equal number of normal cells and the well content was then exposed to PHA. In addition, thymosin and THF were added to appropriate wells at concentrations of 100 and 20 μg/well, respectively.

RESULTS

The PHA mitogenic response of a normal donor's peripheral blood lymphocytes (PBL) was 82,865 net counts per minute (cpm), whereas that of a lymphoma patient was 3932 net cpm. The addition of patient cells to normal ones suppressed the mitogenic response of the latter by > 50%. Thus, the PHA response of the two populations together was 37,651 net cpm (fig 1). Since the cultures were incubated for only 72 hours, little mixed leukocyte reaction was generated and the two populations cultured without mitogen generated 3527 cpm. When the patient PBL were irradiated prior to their addition to the culture, the suppressive effect was totally abolished and the mitogenic response of these two populations was 81,499 net cpm. Pretreatment with THF as described above restored the mitogenic response almost completely (70,699 net cpm), as did thymosin (73,916 net cpm). Incubation of a patient's lymphocytes alone with thymic hormones did not restore the mitogenic response. Thus, THF incubation increases the PHA response of these cells only from 3932 to 5705 cpm, and thymosin increased it only to 9720 cpm. THF caused an increase of 13% in the PHA mitogenic response of normal cells cultured alone while thymosin caused an increase of 30% in the normal PHA mitogenic response. None of these changes in single populations was statistically significant because, in our system, only a 100% increase or a 50% decrease in cpm is significant at the 5% level.

When the same experiment was conducted with two cell populations obtained from normal donors, no suppression was observed (fig 2). Irradiation of one of the populations prior to its addition into the well results in a net decrease of the PHA mitogenic response from 82,865 net cpm in the single culture to 56,968 net cpm in the mixed culture. In vitro treatment with THF causes no change in the mitogenic response of the two combined populations (85,540 net cpm), while thymosin treatment caused an increase to 111,453 net cpm.

The suppressor cell activity in the group of patients studied and its mitigation by irradiation, thymosin, or THF are summarized in table 1. Of the 14 patients with stage IV metastatic cancer studied, 12 had some suppressor cell activity. This suppressor effect was partially mitigated by irradiation in nine of the 14 patients studied. In vitro, thymosin treatment as described above was attempted in ten patients and showed some effect in five, while THF reduced suppressor activity in four of 11 patients. The comparative efficacy of irradiation, thymosin, and THF to reduce the suppression caused by cancer patients' PBL is also shown in table 1. It can

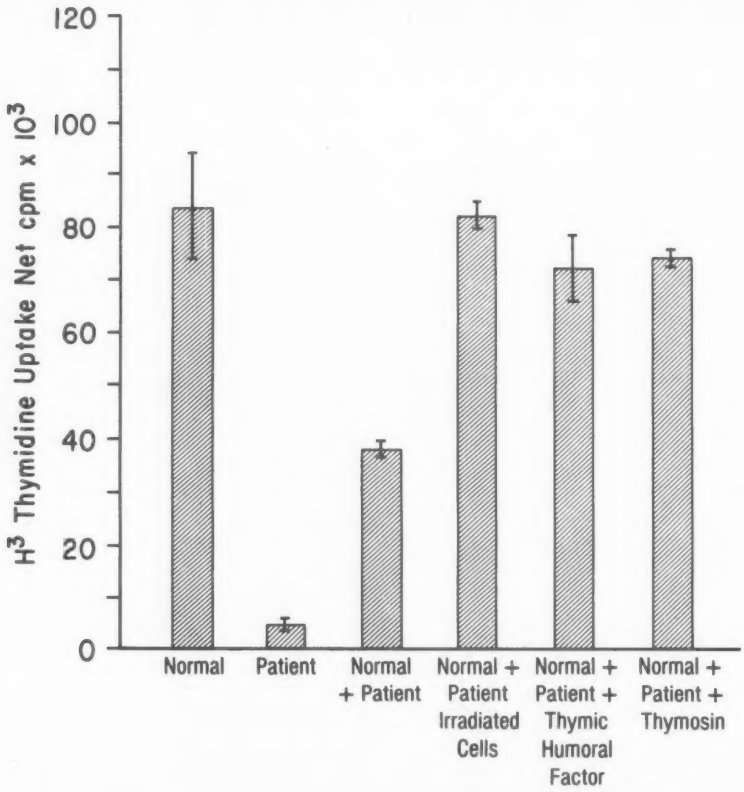

FIGURE 1.—Effect of radiation and thymic hormones on the suppression of the normal lymphocyte PHA response by a cancer patient's lymphocytes.

be seen that all of the cell preparations that showed reduced suppression as a result of thymosin addition did so to THF as well. In one patient's preparation, suppression was reduced by thymosin and not by THF. The incidence of suppressor cell activity and its mitigation by irradiation or by thymic hormones are summarized in table 2.

DISCUSSION

The immune suppression present in the animal (6) and human (4,5) tumor-bearing host can at least in part be attributed to the presence of suppressor cells. A variety of suppressor cells have been identified in cancer patients. A T-suppressor cell (13) as well as a prostaglandin-producing glass adherent cell (12) have been described in a patient with Hodgkin's disease. Nonspecific suppressor cell activity sensitive to carrageenan and thus presumably monocytic in origin was also identified (14).

We have also identified suppressor cell activity capable of significantly decreasing a normal subject's mitogenic response to PHA. This suppressor cell activity, which was identified in 12 of 14 cancer patients, had the following characteristics: (*a*) radiosensitivity: irradiation with 4000 rads reduced the suppressor effect in nine of 14 patients; (*b*) thymic hormone responsiveness: in vitro treatment with thymic hormones followed by the addition of thymosin partially mitigated the suppressor activity in five of ten patients and THF did the same in four of 11 patients.

It is feasible that this suppressor cell activity is responsible for the immunosuppression observed in cancer patients (3,4) since 12 of 14 patients who suppressed the PHA mitogenic response of a normal donor failed also to generate a blastogenic response to that mitogen. Radiosensitivity and thymic hormone responsiveness would tend to identify this cell as a lymphocyte of the T-cell lineage. A radiosensi-

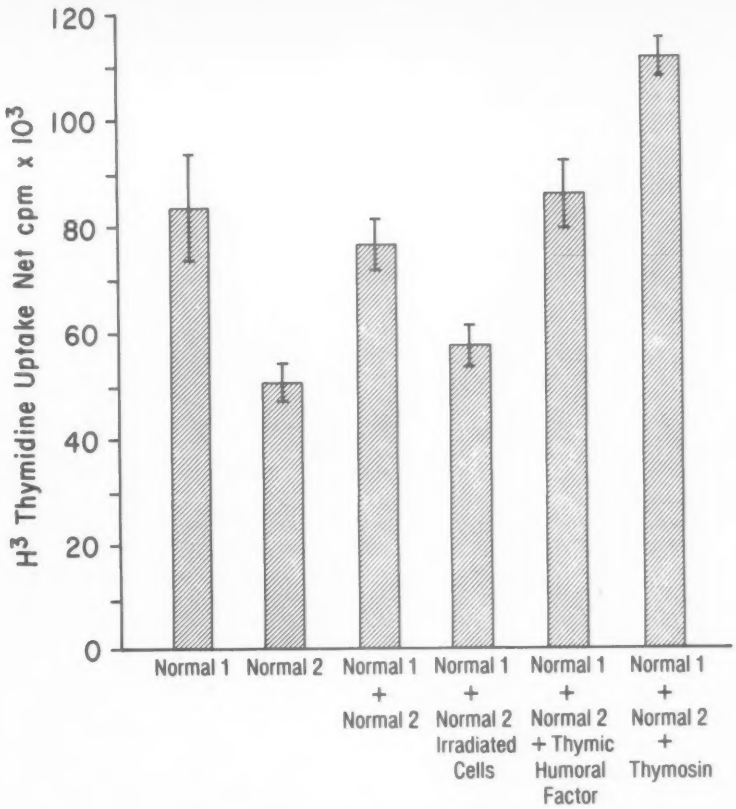

FIGURE 2.—Controls for suppressor cell experiment.

TABLE 2.—Incidence of suppressor cell activity in cancer patients and its mitigation by irradiation, thymosin, or THF

Total No. of patients studied	14
No. of patients with—	
Suppressor cell activity/total studied	12/14
Mitigation of suppressor cells by—	
Radiation/total studied	9/14
Thymosin/total studied	5/10
THF/total studied	4/11

tive T-suppressor cell was described by Engleman et al (22). The radiosensitivity was dose-related and the suppressive activity could only be totally abolished with 6000 rads.

The thymic hormone responsiveness among our patients' cells points to the possibility that in the appropriate cases an immature T cell may have suppressor activity which could be mitigated by in vitro treatment with thymic hormones. Yet, in the murine system, Cantor et al (23) have shown that the Ly 1 subclass of T_1 lymphocytes is triggered to exert antigen-specific helper activities, while the Ly 23 subclass contains a predominant number of cells effecting antigen-specific suppression. The expression of surface antigens is usually associated with more mature populations, while the sensitivity to thymic hormones is usually unique to immature populations. Evidence for the existence of an immature T-suppressor cell can be drawn from Folch and Waksman (24) who demonstrated a cortisone-sensitive glass adherent T-suppressor cell. Small (25) has also demonstrated an immature cortisone-sensitive thymocyte capable of enhancing syngeneic tumor growth. Obviously the characteristics and type of the suppressor cell or cells in these patients will require extensive confirmation of these preliminary results and fractionation and study of the subpopulations among their peripheral blood mononuclear cells.

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Thymosin-Inducible Lymphocytes in the Peripheral Blood of Patients With Malignant Melanoma [1]

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SUMMARY

E rosette-forming (T) lymphocytes and surface immunoglobulin-bearing lymphocytes were estimated in 85 patients with malignant melanoma. The melanoma patient group had lower mean levels of T lymphocytes and higher mean levels of immunoglobulin-bearing (? B) lymphocytes than did normal subjects. The absolute and percentage depressions of T-cell levels in the melanoma patients were stage-related, as was the depression of total lymphocyte and B-lymphocyte levels. The T lymphopenia in the melanoma patients could, in vitro, be partially abolished by fetal calf serum (as used in many E rosetting methods), and could be totally abolished by thymosin fraction 5 (Hoffmann-La Roche) at optimum concentration. In view of the ability of thymosin to restore T cells to normal levels in all of the T-lymphopenic patients, a clinical trial of this hormone in selected melanoma patients of all stages appears to be warranted.

[Cancer Treat Rep 62:1769–1774, 1978]

Patients with advancing malignant neoplasms such as malignant melanoma have depressed cell-mediated immunity (1) and markedly depressed E rosette-forming (T) lymphocyte levels in the peripheral blood (2–4). Indeed, there appears to be a strong correlation between T-cell levels on the one hand and stage of disease and prognosis on the other (2).

The cell-mediated immunity of cancer patients may be further impaired by conventional treatments such as chemotherapy (5) and/or radiotherapy (6). Although radiotherapy may result in a T lymphopenia lasting for a period of years (6), the immunosuppressive effects of surgery appear to be only temporary (7). The present study includes patients treated only by surgery.

E rosetting techniques vary considerably between laboratories, with some investigators including and others omitting fetal calf serum (FCS) from their test systems. FCS enhances E rosette formation by lymphocytes from cancer patients (8), and may result in the failure to detect T lymphopenia in these patients (4,8).

Immunologic intervention, aimed at restoration of T-cell numbers and function, may induce clinical remission. Thymosin, an extract of bovine thymus, significantly increases E rosette formation by T lymphocytes from patients with solid tumors in vitro and in vivo (9,10). Recent results suggest that thymosin also restores T-cell function in cancer patients in vivo (10).

We have investigated the T and B lymphocytes in patients with malignant melanoma, and have compared them with the results obtained from healthy normal control subjects. This particular histologic tumor type was selected for study since host resistance, possibly immunologically determined, appears to be a major factor in determining the prognosis of the melanoma patient. E rosette formation was examined in vitro in medium, medium plus thymosin, and medium plus FCS.

[1]Supported by Hoffmann-La Roche Inc (Nutley, NJ), the Fane and Lawson Trusts, the Cancer Research Campaign, and the Westminster Research Trust.

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[4]We are grateful to Dr. David Preston of the Department of Computer Medicine, Westminster Hospital, for providing computer facilities.

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TABLE 1.—Influence of thymosin and FCS on the distribution of surface markers of peripheral blood lymphocytes from 85 staged malignant melanoma patients and of 50 healthy normal control subjects*

1	2	3	4	5	6	7	8	9	10
Diagnosis	No. of subjects	T cells in medium alone	Maximum T cells with thymosin	Thymosin-inducible T cells (T_i)	B cells	Maximum null cells†	Minimum null cells‡	Optimum thymosin concentration (μg/ml)	T cells in medium plus FCS
Normal controls	50	47.7	62.6	14.9	13.9	38.8	24.0	150	49.2
Melanoma									
Stage I	42	38.4 (0.00013)	61.2 (NS)	22.9 (NS)	28.6 (< 0.00001)	33.8 (NS)	13.8 (< 0.05)	660 (0.05)	48.6 (< 0.016)
Stage II	28	32.1 (< 0.00001)	59.3 (NS)	25.5 (NS)	23.9 (< 0.00001)	45.2 (NS)	15.7 (NS)	272 (NS)	48.4 (< 0.05)
Stage III	15	27.0 (0.00002)	59.2 (NS)	31.9 (NS)	20.3 (0.00002)	52.1 (0.01)	20.1 (NS)	1018 (0.01)	49.4 (NS)
All stages	85	34.2 (< 0.00001)	59.3 (NS)	23.8 (NS)	24.2 (< 0.00001)	41.1 (NS)	16.5 (NS)	594 (0.02)	47.9 (0.0001)

*Figures are in percentages. *P* values are given in parentheses and were obtained by the Mann-Whitney U test. NS = not significant.

†100 – [columns 3 + 6].

‡100 – [columns 4 + 6].

PATIENTS AND METHODS

Patients

Eighty-five patients (54 women and 31 men) with histologically proved malignant melanoma were studied. The classification of Lewis et al (11) was used to assign 42 patients to stage I, nine to stage IIa, 14 to stage IIb, five to stage IIab, and 15 to stage III. The overall age range for patients was 13–80 years (mean, 49). Approximately half the patients were symptom-free and half had active disease at the time of the study. No patient had received radiotherapy or chemotherapy at any time or had received surgery within 2 weeks of the investigation.

The 50 healthy normal control subjects whose age range was 18–50 years (mean, 29) were blood donors or on the hospital staff. They were not matched for age and sex with the patients, but their ages were not significantly different from those of the patients.

Methods

These methods have already been described in detail (4). The thymosin was bovine extract, fraction 5 (Hoffmann-La Roche), batch C 98035.

RESULTS

In table 1, all results are stated as percentages of the separated lymphocytes, so that the sum of columns 3 + 6 + 7 ≃ 100% without added thymosin and columns 4 + 6 + 8 ≃ 100% at the concentration of thymosin producing maximal in vitro increase of T cells. In table 2, the results are stated in terms of absolute numbers of cells/mm^3 of peripheral blood, calculated from the absolute lymphocyte count, and the percentages shown in table 1. In both tables, all melanoma groups have been compared with the control group using the Mann-Whitney U test, and the respective *P* values thus obtained are given in parentheses.

When all of the melanoma patients are considered together, the overall effect of the thymosin can be assessed (table 1). The difference with and without added thymosin represents a large population (about 25%) of thymosin-inducible T cells (T_i) which would normally be classified with the non-T or -B (null) cells.

Four subpopulations of lymphocytes have thus been detected: T cells (independent of added thymosin), inducible T cells (T_i) (dependent upon added thymosin), B cells, and null cells (apparently refractory to thymosin).

The range of optimum thymosin concentrations required for maximal E rosette formation was very wide, ranging from < 0.002 to > 2000 μg/ml (tables 1 and 2). The relationship between thymosin concentration and $\%T_i$ in most patients closely resembled a typical dose-response curve, with actual rosette inhibition at higher concentrations of the hormone. The peak rosette numbers occurred at widely varying concentrations of thymosin.

TABLE 2.—Influence of thymosin and FCS on the distribution of surface markers of peripheral blood lymphocytes from 85 staged malignant melanoma patients and of 50 healthy normal control subjects*

1	2	3	4	5	6	7	8	9	10	11
Diagnosis	No. of subjects	Total lymphocytes	T cells in medium alone	Maximum T cells with thymosin	Thymosin-inducible T cells (T_i)	B cells	Maximum null cells†	Minimum null cells‡	Optimum thymosin concentration (μg/ml)	T cells in medium plus FCS
Normal controls	50	2424	1155	1525	369	342	950	581	150	1226
Melanoma										
Stage I	42	2151 (NS)	818 (NS)	1288 (NS)	482 (NS)	632 (0.00009)	700 (NS)	271 (0.04)	660 (0.05)	1025 (NS)
Stage II	28	1937 (< 0.001)	556 (0.0006)	1117 (< 0.05)	483 (NS)	509 (0.05)	830 (NS)	344 (NS)	272 (NS)	856 (NS)
Stage III	15	1889 (0.004)	619 (< 0.002)	1241 (0.02)	591 (NS)	463 (NS)	969 (NS)	379 (NS)	1019 (0.01)	936 (NS)
All stages	85	2042 (< 0.0005)	694 (< 0.00001)	1209 (NS)	484 (NS)	564 (< 0.00001)	806 (NS)	347 (NS)	594 (0.02)	945 (0.0001)

*Figures are in absolute numbers of lymphocytes/mm^3 of peripheral blood. P values are given in parentheses and were obtained by the Mann-Whitney U test. NS = not significant.

†Total – [columns 4 + 7].

‡Total – [columns 5 + 7].

In general, lymphocytes from normal subjects showed little or no thymosin inducibility in most cases (fig 1); indeed, 25% of the samples tested were inhibited at all concentrations of the hormone, as were the cells from just four of the patients. However, cells from several of the controls showed large increases in T cells by the addition of thymosin.

Correlations With Tumor Stage (tables 1 and 2 and fig 2)

T lymphopenia (lower normal limit, 470 cells/mm^3) was observed in 17 (40%) of the stage I patients, 15 (54%) of the stage II patients, five (33%) of the stage III patients, and one (2%) of the control subjects. If the tests were performed in medium containing FCS, only 12 (32%) of the T lymphopenias in the patient group were detected. If the tests were performed in medium containing thymosin at optimum concentration, no T lymphopenias were detected.

DISCUSSION

We have counted percentages and total numbers of peripheral blood lymphocytes forming E rosettes in melanoma-bearing patients. Both percentages and absolute numbers are diminished as compared with normal controls, in accordance with earlier findings in melanoma (2–4). Indeed, studies of patients with solid tumors almost invariably have shown T-cell depletion in advanced stages of the disease, with a corresponding increase in nonrosetting cells. Our present results indicate that these nonrosetting cells are, in fact, immunologically null lymphocytes, as we have reported earlier (4).

It appears that depressed T-cell levels in the patients are disease-related and not the result of prior treatment, since untreated stage I patients showed T-cell depletion. No patient had received chemotherapy or radiotherapy, both of which may result in low T-cell numbers; indeed, the immunosuppressive effects of chemotherapy or radiotherapy may facilitate tumor proliferation and nullify therapeutic benefits (6). Followup studies on some of our stage I melanoma patients after surgery showed no significant depression of immunologic parameters. Most stage II and III patients had received surgery, with the interval since last surgery ranging from 2 weeks to 14 years (mean, 26 months). The immunosuppressive effects of surgery have been found by numerous investigators to be only temporary and are unlikely to have influenced the results in this study.

The finding of T-cell depletion in cancer patients correlates with other evidence of depressed cell-mediated immunity. Lymphocyte-mediated cytotoxicity (12) and lymphocyte transformation to allogeneic lymphocytes (1), for example, are almost invariably impaired or absent in advanced cases of malignant melanoma.

The concept of immunosurveillance implies that T

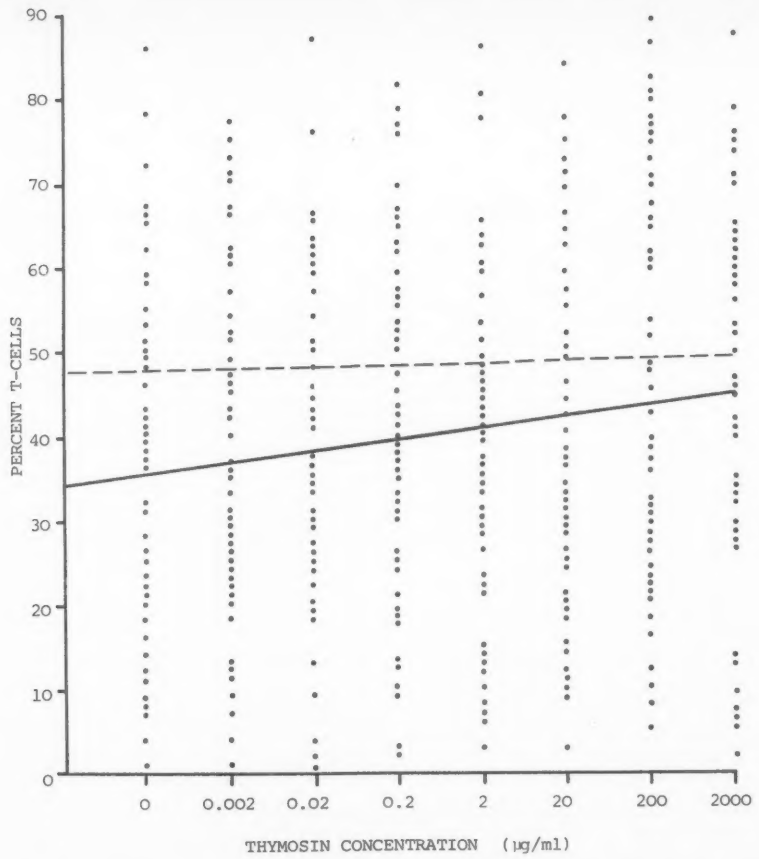

FIGURE 1.—Scattergram showing the distribution of T-cell percentages according to the in vitro thymosin concentration in lymphocyte populations from the peripheral blood of 85 malignant melanoma patients. Linear regression analysis of the data yields a correlation coefficient, r, of 0.13 ($P = 0.01$) for the melanoma group (solid line). The corresponding regression line for the 50 normal control subjects (broken line) has been superimposed upon this scattergram.

cells and other immune mechanisms can destroy emerging clones of malignant cells. In view of this role, T cells are uniquely important, and qualitative and quantitative depletion of T cells might therefore result in the emergence and metastatic spread of malignant cell clones. The increased incidence of tumors in immunosuppressed or immunodeficient patients, and the finding of extremely small (subclinical) tumors in many patients dying from nonmalignant causes, further emphasize the importance of the normal immune response in the suppression of tumors. It appears that the mere presence of the tumor is associated with depressed T-cell levels, although it is unclear whether the presence of the tumor depresses T-cell levels or if low T-cell levels predispose to tumorigenesis. Thus, it is controversial whether the undoubted immunologic defect which exists in melanoma is due to primary thymic abnormality or is secondary to the disease itself.

The results of the present study confirm the stage-related nature of the T-cell depletion in malignant melanoma, as previously described in cancer in general (13), in breast cancer (14), in lung cancer (15), and in malignant melanoma itself (2), and usually with a corresponding increase in the numbers of non-T lymphocytes (14). The T-cell depletion in our malignant melanoma patients is consistent with the stage-related depression of the mixed lymphocyte reaction in melanoma patients reported in an earlier paper from our laboratories (1). The greatest fall in T-cell levels was in stage I pretreatment, suggesting that immunity is impaired early in the disease process and not necessarily as a result of treatment.

In addition to a stage-related fall in T-cell percent-

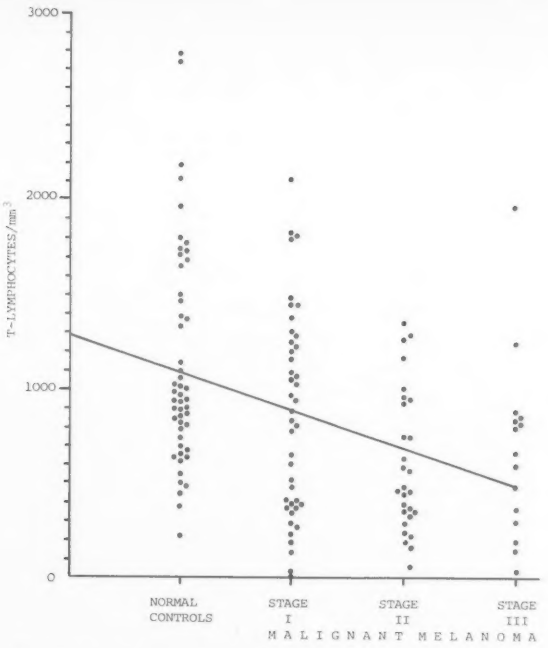

FIGURE 2.—Scattergram showing the distribution of T cells/mm³ of peripheral blood according to stage of disease in 85 malignant melanoma patients. Thymosin and FCS were omitted from the E rosette assay systems. Linear regression analysis of the data yields a correlation coefficient, r, of 0.36 ($P < 0.001$).

ages, we, like Bourgoin et al (2), have also found stage-related depletion in overall lymphocyte levels in our melanoma patients. Similar results have been found in carcinoma of the bronchus (16) and in lung carcinoma (17). Thymic factors are known to stimulate lymphocytopoiesis (18), and the lymphocyte depletion in our melanoma patients may be due to thymic insufficiency or, of course, immunologic exhaustion.

The overall mean level of B lymphocytes (table 1) in our melanoma patients is identical to that reported by Peter et al (3). This mean level was significantly higher than the control group mean level, in accordance with the results obtained in breast cancer (14) and in carcinoma of the bronchus (16). The fall in B-cell levels was stage-related, providing further evidence of immunologic exhaustion.

Having studied the levels of T and B lymphocytes in the patients and in the normal subjects, we can calculate the numbers of null cells (tables 1 and 2). Although 24 of the 42% null cells can be accounted for as T_i cells, the remaining 17% still cannot be ascribed to the T or the B axis. These refractory cells may be stem cells, line progenitor cells, immature lymphocytes, defective or moribund lymphocytes, lymphocytes in active cell cycle, blocked T or B cells, or blocked K ("killer") cells. Our attention, therefore, is drawn to the population of T_i cells, which constitutes no less than one fourth of the entire lymphocyte population in our melanoma patients (as we have already found in hypernephroma and breast cancer patients [4]).

The results support the finding that thymosin significantly increases E rosette formation by T lymphocytes from patients with solid tumors (4,9,10,19). In marked contrast to normal subjects, lymphocytes from 81 of the 85 patients with malignant melanoma contained between 1% and 77% of T_i cells, representing in absolute numbers mean increases from 694/mm³ without thymosin to 1209/mm³ with thymosin. For the control group, the figures are 1155/mm³ without thymosin and 1525/mm³ with thymosin. The majority of melanoma patients appear to behave as if they are thymosin-deficient or -resistant, assuming that it is valid to extrapolate these results to the in vivo situation.

It seems that the increase in T cells after thymosin induction of melanoma lymphocyte populations is due to the conversion of part of the null cell population into E rosetting cells. This might be achieved by the conversion of pre-T cells to T cells, or by the removal of some (? blocking) factor from the surface of otherwise mature T cells. It is quite possible that the T_i cells might have a suppressor cell role which is lost as a result of thymosin treatment. In any case, thymosin induction indicates that these melanoma patients are not permanently T lymphopenic, only reversibly so.

Since thymosin (fraction 5) represents a mixture of polypeptides with a combined action (20) on T_i cells, variation in optimum in vitro thymosin concentrations might be expected, but this of course means that the use of a single concentration of thymosin in an in vitro test can grossly underestimate such T_i cells. It also means that if thymosin therapy is to be scientifically evaluated in cancer patients, then each patient should be tested in vitro over a wide range of thymosin concentrations to select the appropriate dose, if any. Furthermore, similar followup studies after in vivo use of thymosin could indicate whether all of the T_i cells have been converted to E rosetting T cells, and such titration could guide further therapy. If these T_i cells represent young committed or mature but blocked T cells, or if they are all suppressor cells, therapeutic thymosin might encourage their conversion to an anticancer role.

In vivo studies in cancer patients have confirmed the validity of the in vitro E rosette test for predicting which patients might benefit from thymosin. Following a short course of thymosin treat-

ment, the majority of patients whose cells responded in vitro to the hormone showed a significant increase in absolute numbers of T cells in the blood and/or conversion from negative to positive delayed hypersensitivity skin tests (9,10,21). The effect of thymosin was most pronounced if the baseline rosettes were < 50% initially; our own findings agree in principle with these. Although clinical improvements have been noted in a few patients who have received thymosin therapy, it is not yet known if these improvements can be ascribed to thymosin (10).

Of course, in vitro, the separated lymphocytes are washed and are away from the influence of factors in the plasma of cancer patients which are known to inhibit T-cell function (1,22). For this reason, it might be necessary to reduce such inhibitory activity by preliminary plasma exchange (23) before inducing T cells with therapeutic thymosin.

In conclusion, these results emphasize the prognostic importance of the maintenance of T-cell levels. A marked collapse in T-cell levels commonly preceded clinical deterioration in a series of bronchus carcinoma patients (16). Thus, it appears that low T-cell levels are secondary to the presence of the tumor. Impaired cell-mediated immunity has been correlated with a poor prognosis in cancer (24), while restoration of cell-mediated immunity (25) and of T-cell levels (14,15) is often associated with successful treatment.

In the present study, those patients with low absolute levels of T cells have an increased number of thymosin-inducible cells, and the ability of thymosin to raise the levels of T cells to normal in vitro may represent a method of correcting this deficiency. Since FCS has thymosin-like activity, its use in E-rosette tests such as the Wara-Ammann assay (26) should be reviewed.

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Effect of Thymosin and Irradiation on Immune Modulation in Head and Neck and Esophageal Cancer Patients [1]

William M. Wara,* Arthur J. Ammann, and Diane W. Wara [2]

SUMMARY

Fifty-five patients with squamous cell carcinoma of the head and neck and esophagus were evaluated prior to irradiation and thymosin fraction 5 therapy. Immunity prior to treatment, as measured by total lymphocyte count, E and EAC rosettes, lymphocyte stimulation with phytohemagglutinin (PHA) and in mixed leukocyte culture (MLC) with allogeneic cells, delayed hypersensitivity skin tests, and quantitative serum immunoglobulins, was comparable and normal in the 40 control patients and in the 15 thymosin-treated patients. After irradiation, significant depression ($P < 0.01$) was demonstrated in cellular immunity in both groups of patients with decreased T- and B-cell numbers and depressed phytohemagglutinin and MLC stimulation. Six months after irradiation, our preliminary results suggest that the thymosin-treated patients may be reversing their immunosuppression by a return of MLC function and positivity of delayed hypersensitivity skin tests. The ultimate effect of thymosin on disease control and survival remains uncertain.

[Cancer Treat Rep 62:1775–1778, 1978]

The prognosis of squamous cell carcinoma of the head and neck and esophageal region is poor (1). Standard therapy for the disease, irradiation of the primary tumor and adjacent lymph node areas, causes suppression of cell-mediated immunity. We elected to evaluate prospectively the effect of immune modulation in this group of patients.

Previous data from patients with nasopharyngeal carcinoma treated with radiation fields similar to most head and neck cancer patients indicated that the thymus received a minimum dose of approximately 390 rads of irradiation which resulted in immunosuppression (2). Recent studies by Dellon and associates (3) and Stratton et al (4) indicate that irradiation to the mediastinum or pelvis causes a rapid decrease of both B (bone marrow-derived) and T (thymic-derived) lymphocytes, as measured by E and EAC rosette formation. The effect of this decrease in cell-mediated immunity with regard to prognosis and survival has not been determined. It is possible that this immunosuppression may facilitate growth of primary tumors and/or growth of occult metastases.

These hypotheses and the recognition of the thymus gland as an organ which produces humoral factors which can restore cellular immunity in thymus-deprived mice (5) have led to the investigation of cancer patients receiving mediastinal irradiation. Thymosin fraction 5, which contains at least 12 polypeptides, is one of several extracts isolated from thymic tissue which has been shown to have immune-enhancing capabilities (6). This phenomenon has been observed in primary immunodeficiency disease (7) and in various malignancies (8–11). Therefore, thymosin was added to the standard therapeutic regimen for the patient receiving irradiation for squamous cell carcinoma in an attempt to stimulate uncommitted T cells to mature into functional cells and to determine whether thymosin can alter the disease-free interval, survival, and/or local tumor control rate.

[1]Supported by Public Health Service contract CB-64004 (National Cancer Institute) and a grant from the Division of Research Resources, National Institutes of Health, Department of Health, Education, and Welfare. W. M. Wara is the recipient of an American Cancer Society Junior Faculty Fellowship Award.

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TABLE 1.—Total lymphocyte and E rosette subpopulation in head and neck and esophageal cancer patients before and after irradiation

	Before irradiation*	After irradiation*		
		Immediate	3 mos	6 mos
Patients receiving thymosin				
Total lymphocyte count/mm³ †	1602 (15)	836 (11)	654 (7)	1046 (3)
E cells (%) ‡	71	74	67	73
Total E cells/mm³	1141	618	336	757
Patients not receiving thymosin				
Total lymphocyte count/mm³	1652 (40)	737 (36)	1080 (18)	1080 (15)
E cells (%)	73	77	76	79
Total E cells/mm³	1214	569	821	850

* Nos. in parentheses = No. of patients evaluated.
† Normal = > 1200 cells/mm³.
‡ Normal = > 62%.

MATERIALS AND METHODS

Immunologic Evaluation

An immunologic evaluation was performed in 55 patients who presented to the Division of Radiation Oncology, University of California, San Francisco, for treatment of squamous cell carcinoma of the head and neck and esophagus. After thymosin became available, patients were randomized at presentation to receive or not to receive the drug during their radiation therapy and until recurrence or to a maximum of 1 year. Using methods previously described (12), patients were evaluated with complete blood cell counts, T-cell rosette formation, and lymphocyte stimulation with phytohemagglutinin (PHA) and in mixed leukocyte culture (MLC) before irradiation, after irradiation, and continuing at 3-month intervals for 1 year. Intradermal skin test antigens including purified protein derivative, streptokinase-streptodornase (SK-SD), and *Candida* were placed before and after irradiation. The evaluation also included quantitative immunoglobulins and B-cell number as identified by EAC rosette formation.

Thymosin Administration

All thymosin was obtained from Hoffmann-La Roche, Inc, and thymosin fraction 5 was utilized. After an initial intradermal test dose of 1 mg, thymosin was administered in ten treatments of 40 mg/m² given sc. Subsequently, a dose of 40 mg/m² was given once each week for 50 weeks or until definite evidence of tumor recurrence appeared.

RESULTS

As shown in table 1, before irradiation all patients had a normal number of total lymphocytes and total T cells. There was no significant difference ($P < 0.01$) between the thymosin-treated and untreated patients. There was also no difference between the two groups immediately after, at 3 months after, or at 6 months after irradiation. Both groups were statistically different ($P < 0.01$) with regard to preirradiation values, demonstrating the immunosuppressive effect of the irradiation. However, the percentage of E rosette cells did not change and only a relative lymphopenia was demonstrated.

Table 2 demonstrates that both thymosin and control patients had comparable lymphocyte stimulation with PHA prior to irradiation. In both groups, there was significant suppression after irradiation. Six months after irradiation in a small number of patients there was a suggestion of recovery of function in the thymosin-treated patients.

More striking is the recovery at 6 months of MLC function in the thymosin-treated patients (table 3). Again there was significant suppression after therapy but with a return to pretreatment levels in the thymosin group 6 months after irradiation.

Table 4 shows the results of delayed hypersensitivity skin tests before and after irradiation. Approximately 40% of all patients were anergic to all skin tests prior to irradiation with no difference when re-tested after irradiation. When patients who received thymosin were separated from the total group it is interesting that none was anergic after irradiation with the most significant change, ie, conversion to positivity, occurring with SK-SD.

EAC rosettes showed no change before (17%) and

TABLE 2.—Lymphocyte stimulation with PHA before and after irradiation*

	PHA stimulation (cpm: maximum stimulation)†			
	Before irradiation	After irradiation		
		Immediate	3 mos	6 mos
Patients receiving thymosin	32,433 (14)	12,481 (11)	12,147 (6)	26,136 (3)
Patients not receiving thymosin	26,241 (39)	11,860 (29)	17,176 (17)	21,352 (16)

*Normal = > 44,000 cpm maximum stimulation.
†Nos. in parentheses = No. of patients evaluated.

TABLE 3.—Mixed leukocyte reaction with allogeneic cells before and after irradiation*

	MLC (cpm: maximum stimulation)†			
	Before irradiation	After irradiation		
		Immediate	3 mos	6 mos
Patients receiving thymosin	12,886 (13)	4882 (11)	4610 (5)	8901 (3)
Patients not receiving thymosin	8711 (36)	4089 (30)	5319 (17)	1762 (17)

*Normal = > 6000 cpm maximum stimulation.
†Nos. in parentheses = No. of patients evaluated.

TABLE 4.—Delayed hypersensitivity skin test results before and after irradiation

	Before irradiation	After irradiation
Total patients negative to all tests	39%	45%
Patients not receiving thymosin negative to all tests	40%	37%
Patients receiving thymosin—		
Negative to all tests	22%	0
Negative to *Candida*	73%	57%
Negative to SK-SD	53%	14%

after (16%) irradiation in the thymosin group versus 15% and 16% in the control group. Serum immunoglobulins remained unaffected by treatment. The mean IgG value prior to irradiation was 1110 ± 490 mg/dl, compared to 1158 ± 587 mg/dl after irradiation. The mean IgM prior to irradiation was 172 ± 85 mg/dl compared to 147 ± 61 mg/dl after irradiation. The mean IgA concentration prior to treatment was 275 ± 113 mg/dl, compared to 272 ± 114 mg/dl after irradiation.

DISCUSSION

These results clearly indicate that localized high doses of radiation produce suppression of the immune system at the completion of irradiation (approximately 6 weeks after initiation of therapy). Although the exact mechanism for this depression is controversial, it most likely results from the irradiation of large blood volumes in the treatment fields. In contrast, Stjernsward et al showed minimal suppression in brain tumor patients who have a low blood flow per minute (13). Serum factors have also been implicated in some malignancies with depression of in vitro lymphocyte tests (14). Finally, Law et al and others have demonstrated immunosuppression in malnourished cancer patients which may be an additive factor in our specific group of patients (15).

Various authors have previously described patients with advanced squamous cell carcinoma of the head and neck as having abnormal numbers of T cells and/or decreased lymphocyte stimulation with PHA and in MLC (16–20). These studies have usually examined only one parameter and have lacked sequential studies to document the kinetics of immunologic recovery. In contrast, our patients are relatively normal before irradiation except for a slight depression of PHA stimulation. It is only after irradiation that we demonstrate marked immunosuppression although the patients are not clinically as abnormal as their studies indicate.

Patient compliance and reliability have been problems with a program requiring weekly injections in a group of patients with advanced cancer, low socioeconomic status, and usually chronic alcohol abuse. Side effects to date have been minimal with only two patients being withdrawn from thymosin

treatment after severe erythema and swelling at the injection site. No systemic reactions have been encountered. Our original decision to use a thymosin dose of 40 mg/m^2 was based on pediatric immunodeficiency experience, and since we are early in our randomization we now propose to increase our thymosin dose to 60 mg/m^2 and give our maintenance dose twice weekly.

The kinetics of return of immunocompetence in our cancer patients seem to be parallel to the group of pediatric immunodeficient patients treated with thymosin (21). Not until 6 months after irradiation and after initiation of thymosin therapy do we begin to see recovery of MLC function. Our data (22) and those of Raben et al (21) and Stratton et al (4) suggest an increased proportion of "null cells" (lymphocytes with neither T- nor B-cell surface markers) after irradiation. A logical approach to an immunosuppressed patient after treatment would be to stimulate these "null cells" to immunologically functional cells.

Our preliminary results to date suggest that thymosin may be reversing the documented immunosuppression after irradiation with a latent interval of at least 6 months. The incidence of infection was not increased. The ultimate effect in relation to disease control and survival remains uncertain. To date there have been an equal number of recurrences and deaths in both groups of patients.

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Experiences With Thymosin in Primary Immunodeficiency Disease

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SUMMARY

Thymosin has no effect in vitro on cyclic AMP levels in thymocytes. However, when thymosin was injected into patients lacking "serum factor" (SF) activity, it induced the appearance of SF or SF-like activity and the disappearance of target cells for SF among the peripheral blood lymphocytes of the treated patients. On the other hand, when thymosin was injected into one patient with normal SF activity, it induced a marked decrease of SF activity and the appearance of target cells for SF among the peripheral blood lymphocytes of this particular patient.

[Cancer Treat Rep 62:1779–1785, 1978]

By means of direct measurements of intracellular levels of cyclic AMP (cAMP) in human and mouse thymocytes, we have demonstrated the existence in human serum of a thymus-dependent factor (SF) (1). The target cells for SF were restricted to thymocytes: no effect could be observed on normal human peripheral blood lymphocytes. SF has no effect on other lymphoid and nonlymphoid target cells and the activity on thymocytes is restricted to hydrocortisone-sensitive cells (2), ie, to precursors of mature T cells (3). In contrast to the normal situation, target cells for SF were found to be present among peripheral blood lymphocytes of some patients with primary immunodeficiency disease.

Thymosin has no effect in vitro on cAMP levels in thymocytes (4,5). However, when thymosin was injected into patients lacking SF, it induced the appearance of SF or SF-like material which increases cellular cAMP levels in thymocytes in vitro (5).

In this paper we report the effect of thymosin administration on SF activity and target cells among peripheral blood lymphocytes in five patients.

MATERIALS AND METHODS

Thymosin

Thymosin fraction V, "clinical quality," was prepared according to the procedure of Hooper et al (6) by Hoffmann-La Roche, Inc (Nutley, NJ).

Preparation of Serum Factor

Human blood was collected from the cubital vein and defibrinated by shaking for 8 minutes with glass beads (one bead per 2 ml of blood). The defibrinated blood was transferred to a plastic tube (2070, Falcon Plastics, Oxnard, Calif), kept in melting ice, and centrifuged at 4°C for 15 minutes at 600 × g in order to obtain serum. Seven ml of serum was filtered through an Amicon Centriflo CF 50A (Amicon, Oosterhout, The Netherlands) membrane cone filter (nominal molecular weight cut-off, 50,000) by centrifugation at 4°C for 1 hour at 600 × g.

The whole procedure was performed within 2 hours after blood collection. The ultrafiltrate was divided into appropriate aliquots, frozen at –100°C, and lyophilized.

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Preparation of Mouse Thymocytes

C57BL/6J mice (6–8 weeks old) were used in all experiments. The mice were killed by cervical dislocation and the thymus was removed immediately. These organs were aseptically collected in plastic Petri dishes (3003, Falcon Plastics) containing Earle's balanced salt solution (BSS) (01–39110, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) and were gently pressed through a metal sieve. The cell suspension was passed at 20°C through a 110 × 7-mm glass column containing 25 mg of Leuko-Pak nylon wool (Fenwal Laboratories, Division of Travenol Laboratories, Inc, Morton Grove, Ill) in order to remove aggregates. The column was washed three times with Earle's BSS and the eluted cells were washed twice in the same solution. The cells were counted with a Coulter counter, model ZF, at the appropriate settings. The cell viability, measured by means of trypan blue exclusion, was always > 95%.

Preparation of Human Peripheral Blood Lymphocytes (PBL)

Human PBL were isolated from defibrinated blood by means of conventional buoyant-density centrifugation, washed twice in Earle's BSS, and resuspended in the same medium.

Assay for Serum Factor and Thymosin

Thymocyte suspensions were prepared as described above. The SF preparations to be tested were reconstituted to the original volume of the ultrafiltrate with distilled water and then mixed with an equal volume of thymocytes (final concentration, 2×10^6/ml) in Earle's BSS. Thymosin was diluted with Earle's BSS and mixed with thymocytes to the desired concentration. The final cell concentration was always 2×10^6/ml. Each assay tube contained 5 ml of such mixture. After 5 minutes of incubation at 37°C, the tubes were centrifuged at 4°C for 5 minutes at 600 × *g*. The cell viability, both before and after incubation, was > 95%.

The supernatant was discarded in order to measure the intracellular cAMP only and the pellet was resuspended in 1 ml of 50 mM Tris-HCl/4 mM EDTA buffer, pH 7.5, on a Vortex mixer. The tubes were placed in liquid nitrogen for 1 minute and subsequently in boiling water for 2–3 minutes. Coagulated proteins were spun down at 4°C for 5 minutes at 1500 × *g*. cAMP was purified and measured as reported below. The results were expressed in pmols of cAMP/10^7 thymocytes. Net values were obtained by subtracting the background cellular levels of cAMP (ie, the level of cAMP in thymocytes incubated without SF).

Assay for Target Cells Among Peripheral Blood Lymphocytes

Human PBL suspensions were prepared as described above. The lymphocyte concentration was adjusted to 4×10^6/ml in Earle's BSS. Each "test" tube contained 0.5 ml of such suspension and 0.5 ml of "pool" SF, prepared from the blood of healthy human donors which was tested as described above and found to be active. All assays reported in this paper were performed with "pool" SF of similar activity. Each "control" tube contained 0.5 ml of lymphocyte suspension from the same individual as in the "test" tube and 0.5 ml of Earle's BSS.

After a 5-minute incubation at 37°C, the tubes were centrifuged at 4°C for 5 minutes at 600 × *g*. The cell viability, both before and after incubation, was > 95%. The supernatant was discarded in order to measure the intracellular cAMP only and the pellet was resuspended in 1 ml of 50 mM Tris-HCl/4 mM EDTA buffer, pH 7.5, on a Vortex mixer. The tubes were placed in liquid nitrogen for 1 minute and subsequently in boiling water for 2–3 minutes. Coagulated proteins were spun down at 4°C for 5 minutes at 1500 × *g*. cAMP was purified and measured as reported below. Target cells were scored as "present" when the amount of cAMP that was found in the "test" tube exceeded by a factor of 5 the within-assay coefficient of variation (ie, exceeded by 45% the amount of cAMP found in the "control" tube).

Cyclic AMP Method

After spinning down coagulated proteins, the supernatant was applied to chromatography columns containing 1 ml of Bio-Rad AG 1-X8 (200–400 mesh, formate ionic form) cation exchange resin, equilibrated with 0.1 N formic acid. Unbound compounds were washed away with 10 ml of 0.1 N formic acid. cAMP was eluted with 10 ml of 2 N formic acid, as reported by Kuehl et al (7). The samples were lyophilized to remove formic acid and were reconstituted with 0.2 ml of 50 mM Tris-HCl/4 mM EDTA buffer, pH 7.5.

cAMP was measured by Gilman's competition binding procedure (8), utilizing a binding protein isolated from bovine skeletal muscle. The assay was performed with a cAMP kit (TRK 432, The Radiochemical Centre, Amersham, Great Britain). Each determination was performed in duplicate, with a within-assay coefficient of variation of < 9% (the

between-assay coefficient of variation was < 12%). The results were expressed in pmols of cAMP/10^7 cells. Treatment with phosphodiesterase (Sigma Chemical Co, St. Louis, Mo) confirmed that the material measured was > 90% cAMP.

PATIENTS

Patient 1, the first child of healthy unrelated parents, was hospitalized at the age of 2 years with longstanding diarrhea caused by a *Salmonella typhimurium* infection. At that time, combined immunodeficiency was diagnosed. This could be concluded from the presence of severe lymphopenia (500 cells/μl), the low percentage of T cells (20%), the diminished response of the lymphocytes to mitogenic and allogeneic stimulation in vitro (~ 40% of normal controls), and no response to dinitrochlorobenzene (DNCB) challenge in vivo after appropriate sensitization. Serum IgM, IgA, and IgD were not detectable and the serum IgG (about 62 IU/ml) consisted predominantly of IgG_1 Gm(f) type λ molecules. Following booster, specific antibodies against anamnestic antigens were not present. B lymphocytes were not present in the peripheral blood and bone marrow.

Patient 2, a female infant of healthy unrelated parents, had decreased serum calcium (1.50 mmols/liter) and elevated serum phosphorus (3.28 mmols/liter) in the neonatal period. The absence of a thymic shadow, the low number of lymphocytes (2000/μl), the low percentage of T cells (20%), and the low response of the lymphocytes to mitogenic and allogeneic stimulation (5%–25% of normal controls) sustained the diagnosis of DiGeorge's syndrome. The serum immunoglobulins were of restricted heterogeneity. The level of IgG was 76 IU/ml, the level of IgM was 404 IU/ml, the level of IgA was 15 IU/ml, and the level of IgD was not detectable. A fibrous web was observed between the vocal cords and the presence of an arteria lusoria was diagnosed.

Patient 3, a 9-year-old boy of healthy unrelated parents, was apparently healthy until the age of 7. At that time he was hospitalized for lymph node enlargement with fever, which was shown to be due to lymph node mycosis by *Cladosporium bantianum*. Subsequently, a cellular immunodeficiency was diagnosed on the basis of lymphopenia (1800 cells/μl), the low percentage of T cells (33%), the diminished response to mitogens (50% of normal controls), and the absence of a response in vitro to *Candida albicans*. Serum immunoglobulins and the percentage of B cells were within the normal range.

Patient 4, a 5-year-old boy of Turkish origin, was hospitalized for subacute sclerosing panencephalitis (stage 2) (9). The diagnosis was based on the characteristic clinical and serologic findings and the specific pattern on the electroencephalogram. The titers of measle virus antibodies were high, both in serum (1:1024) and in cerebrospinal fluid (1:32). No abnormal immunologic parameters were found, except a lack of response to DNCB challenge.

Patient 5, a male infant of healthy unrelated parents, was hospitalized at the age of 9 months for longstanding diarrhea. At that time, severe combined immunodeficiency disease was diagnosed from the presence of severe lymphopenia (100 cells/μl), the low percentage of T cells (4%), the absence of a response in vitro to mitogens, the very low response to allogeneic cells (10%), and the low level of serum immunoglobulins (IgM, 60 IU/ml; IgG, 10 IU/ml; and IgA, not detectable). Following nursing in strict reverse isolation and after successful gastrointestinal decontamination, the patient's general condition became reasonably good, but his immune functions remained unchanged.

RESULTS

Table 1 shows that thymosin does not induce an increase in cAMP when tested directly on mouse thymocytes in vitro. In contrast, SF induced a marked increase in cellular cAMP levels. When thymosin was added together with SF, the cAMP levels were the same as with SF alone.

Figure 1 shows the activity of SF before, during, and after thymosin treatment of patient 1. The activity of SF was very low before thymosin administration, reached normal values during treatment, and dropped back to very low values when thymosin was discontinued. Three determinations were performed on the fourth week. The lowest value represents the response occurring 1 hour before the first injection of thymosin. The two identical, and near-normal values, occurred 3 hours after the first injection of thymosin and 5 minutes before the second injection of thymosin. Thymosin treatment

TABLE 1.—Effect of thymosin on cAMP level in thymocytes*

Substances added	pmols of cAMP/10^7 thymocytes
None	2.4 ± 0.2
Thymosin (0.0001–800 μg/ml)	2.3 ± 0.5–3.4 ± 0.8
SF (1:2)	38.7 ± 2.5
SF (1:2) + thymosin (100 μg/ml)	37.7 ± 1.1

* Results are expressed as mean ± SEM of ≥ 4 experiments.

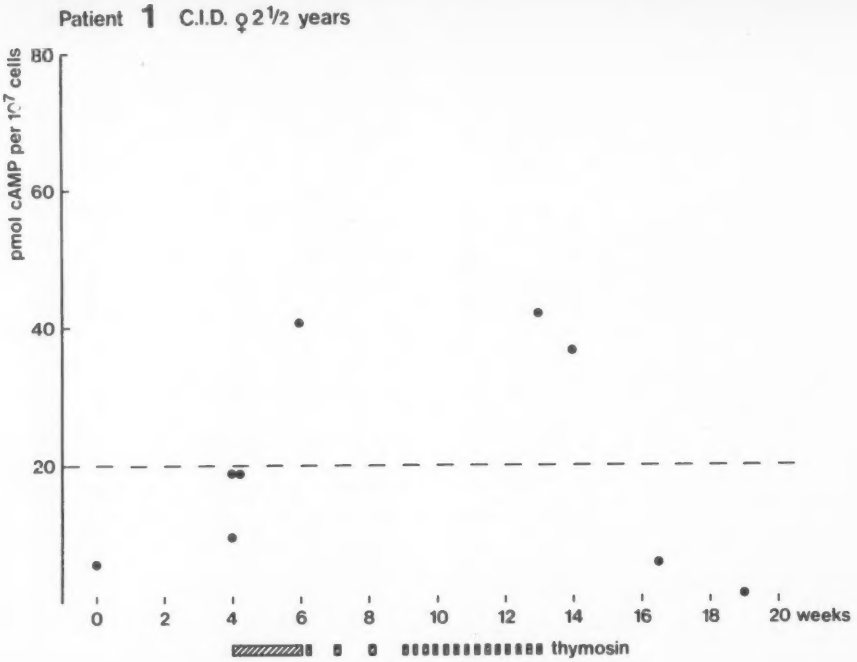

FIGURE 1.—SF activity in patient 1. The horizontal broken line = the lower level of normal values for her age. Thymosin (1 mg/kg of body weight given sc) was administered daily × 2 wks, then once a wk for 3 wks, and subsequently every other day. Treatment was stopped at 13 wks.

had no clinical benefit in this patient. No clear changes in the immune functions could be observed during treatment, except that the percentage of T cells was higher (40%).

Figure 2 shows that patient 2 also had very low SF activity, which reached normal values during thymosin treatment. This patient received three fetal thymus transplants at 0.5, 2.5, and 3 months after hospitalization respectively. No effect of these transplants could be observed on the SF activity and on the immune functions. At 4 months, thymosin treatment was started. At 5 months, the SF activity was normal, the total lymphocyte count was about 6000/μl, and the number of T cells and the lymphocyte response in vitro to mitogens and allogeneic cells progressively increased, reaching normal values a few weeks later. In addition, the clinical condition progressively improved. When thymosin was discontinued the SF activity remained normal. All immune functions also remained normal, and the patient was discharged at 13 months. A biopsy at the site of one of the grafted thymuses showed the histologic picture of a normal thymus. Repeated controls (the last performed at 18 months) showed a clinically healthy child with normal immune functions and normal SF activity, but still retarded in physical and mental development. Target cells for SF were present in this patient's PBL before thymosin administration, were absent during thymosin administration, and reappeared for a short period after thymosin was discontinued. Thereafter, target cells disappeared and remained absent.

Figures 3 and 4 show the SF activity in patients 3 and 4 respectively. In these patients also the SF was low before thymosin administration and normal during treatment. When tested (patient 4, fig 4), target cells for SF were present among PBL before thymosin administration. During treatment, target cells were absent with the exception of one determination just after the start of the second course of thymosin. Except that the DNCB skin test became positive in patient 4, no clear changes in the immune functions were observed and clinically both patients deteriorated rapidly.

Patient 5 showed a normal SF activity at presentation (fig 5). Target cells for SF were absent in the PBL. At 4 weeks, he received a transplantation of fetal liver cells. Eight weeks later the percentage of T cells was normalized (~ 60%), the SF activity

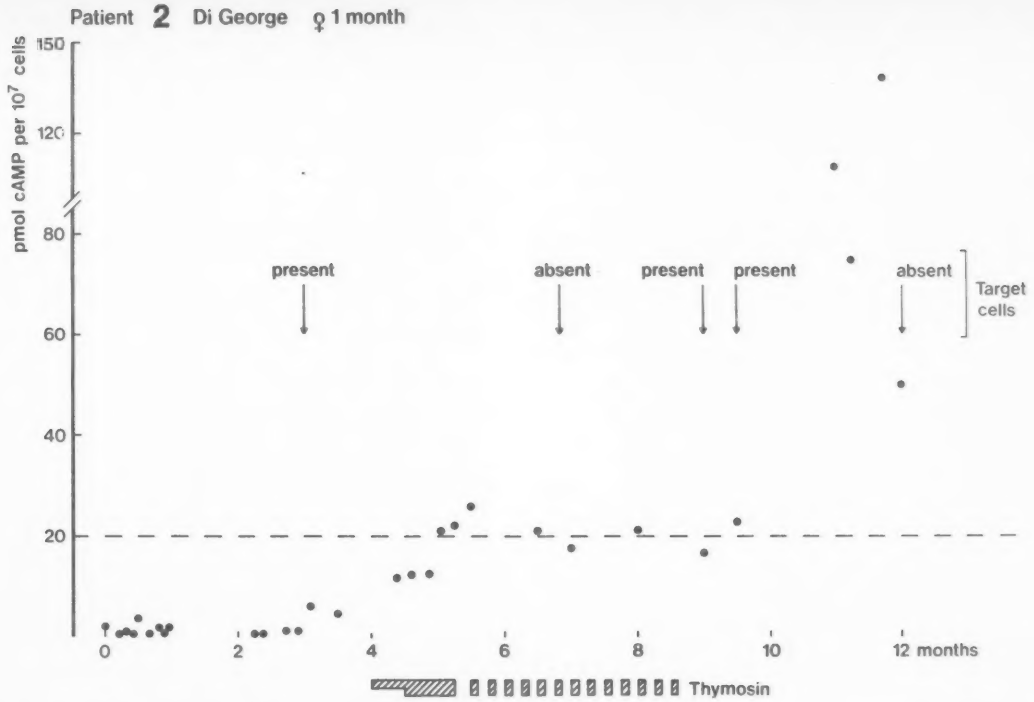

FIGURE 2.—SF activity and target cells among PBL in patient 2. The horizontal broken line = the lower level of normal values for her age. Thymosin was administered daily × 5 wks (1 mg/kg of body weight given sc the first 2 wks and thereafter 2 mg/kg of body weight). Subsequently, thymosin (2 mg/kg of body weight) was given once a wk for 14 wks. Before thymosin therapy was started in this patient, 3 fetal thymuses were transplanted at 0.5, 2.5, and 3 mos respectively.

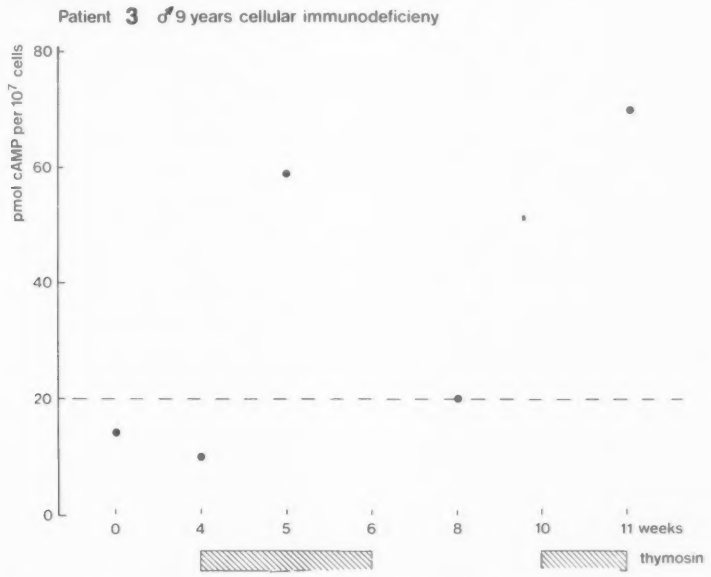

FIGURE 3.—SF activity in patient 3. The horizontal broken line = the lower level of normal values for his age. Thymosin (1 mg/kg of body weight given sc) was administered daily × 2 wks and later for 1 additional wk.

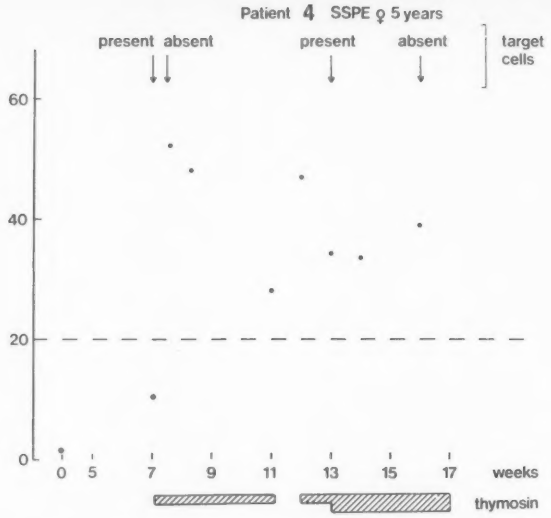

FIGURE 4.—SF activity and target cells among PBL in patient 4. The horizontal broken line = the lower level of normal values for her age. Thymosin (1 mg/kg of body weight given sc) was administered daily × 4 wks and, after a short intermission, daily for 5 additional wks (1 mg/kg of body weight the 1st wk and thereafter 2 mg/kg of body weight).

remained normal, and the target cells in PBL remained absent. The absolute number of lymphocytes was, however, still lower than normal and the lymphocyte response to mitogens and allogeneic cells remained very low. A split-chimerism could be proven by HLA typing, with T cells of donor origin and B cells of patient origin. In view of the lack of lymphocyte function, thymosin was administered beginning at 35 weeks. During the period of daily thymosin administration, the SF activity dropped below normal, the target cells for SF appeared among the PBL, and the percentage of T cells dropped to 30%. When thymosin was administered once a week, the SF activity returned to normal, the target cells disappeared from the PBL, and the percentage of T cells increased slowly. IgG and IgA remained low and IgM decreased to 15 IU/ml. The T cells remained of donor origin and lymphocyte function remained very low. Clinically, no clear changes were observed, but it should be taken into account that, as mentioned above, the general condition of the patient was already reasonably good.

DISCUSSION

As our results show, thymosin has no effect on cellular levels of cAMP in vitro. However, when thymosin is injected into patients lacking SF, it induces the appearance of SF or SF-like activity in the serum of treated patients. Thymosin also induces the disappearance of target cells from the PBL of such patients. The prompt appearance of SF activity after thymosin administration and its disappearance when treatment was stopped in patient 1 indicate that the presence of SF is indeed related to the thymosin treatment. However, as discussed in a previous paper (5), the fact that SF activity is induced by thymosin does not necessarily imply that SF is derived from the thymosin molecule. Thymosin could also act on certain organs or cells and induce them to produce or release factors. We currently favor the latter hypothesis, since the results shown in this paper support this idea. In fact, patient 2 retained SF activity also after thymosin was discontinued. Since a normal SF activity is consistently found in this patient, even at 9 months after the last injection of thymosin, it seems almost inconceivable that the SF activity in her serum might be due to residual thymosin.

In addition, when thymosin was injected into patient 5, we observed a dramatic drop of SF activity. This might be explained on the basis of a thymosin-induced negative feedback on the production or release of SF. If, on the other hand, SF was derived from the thymosin molecule, we should have found in the blood of this patient at least the SF activity derived from the administered thymosin. We conclude that thymosin and SF are most likely two distinct entities and that thymosin regulates the production or release of SF. In view of the findings observed in patient 5, it also seems likely that the presence of target cells for SF among PBL is regulated by the activity of SF rather than by thymosin.

As far as the clinical effects of thymosin are concerned, we do not have evidence for any clinical benefit except in patient 2. And even in this patient we cannot conclude that the observed benefit is entirely due to thymosin, since the patient had received three thymus transplants which, possibly in combination with thymosin, might be responsible for the patient's recovery. This is also supported by the normal thymic tissue found in the biopsy. The improvement observed in this patient is most likely due to the improvement in the function of her own lymphocytes since there has been no evidence of lymphoid chimerism or of graft-versus-host reaction.

It should, however, be mentioned that this report concerns a very limited group of patients and that other reports (10,11) describe a beneficial effect of thymosin in certain primary immunodeficiency diseases. Large-scale trials, now being performed on a multicenter basis, may give a better insight into the actual role of thymosin in the treatment of primary immunodeficiency diseases.

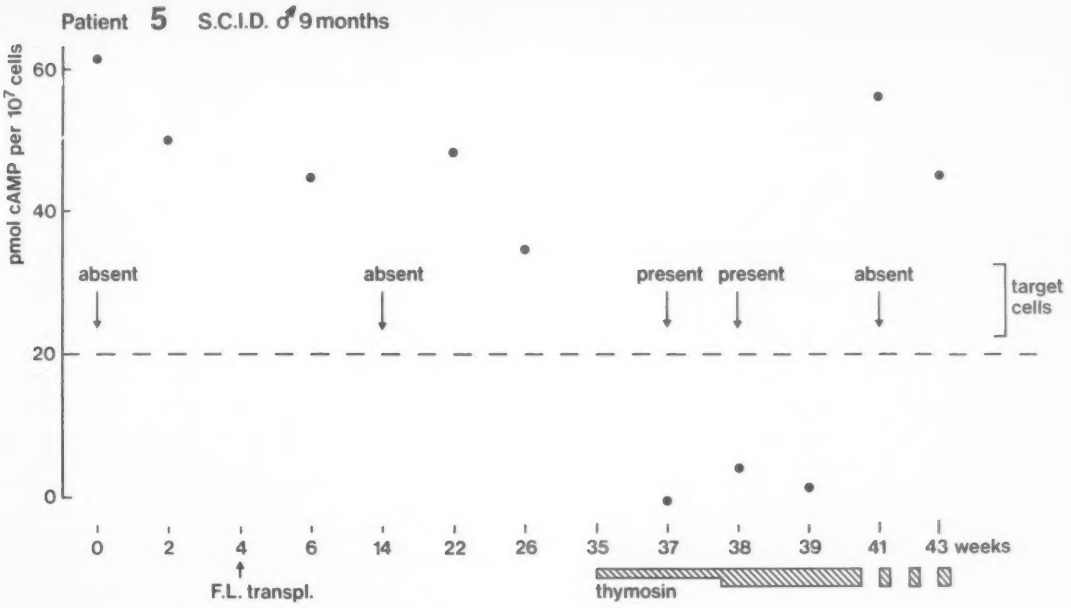

FIGURE 5.—SF activity and target cells among PBL in patient 5. The horizontal broken line = the lower level of normal values for his age. Thymosin (1 mg/kg of body weight given sc for the first 2 wks and thereafter 2 mg/kg of body weight) was administered daily × 4 wks and thereafter once a wk. This patient is still receiving thymosin at the date of the preparation of this paper. Before thymosin therapy was started, this patient received a transplant of fetal liver cells at 4 wks.

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Thymosin in Cancer Patients: In Vitro Effects and Correlations With Clinical Response to Thymosin Immunotherapy

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SUMMARY

Studies on the effect of thymosin on T-cell levels in vitro among normal persons and cancer patients show that, in general, T-cell levels increase after incubation with thymosin in populations with low initial T-cell levels while the levels decrease in populations with high initial T-cell levels. In patients with small cell carcinoma of the lung receiving intensive chemotherapy also randomized to receive thymosin at a dose of 60 mg/m^2, thymosin at a dose of 20 mg/m^2, or placebo twice weekly, increased survival occurred in patients receiving the thymosin dose of 60 mg/m^2. The increase in survival was greatest in patients with low pretreatment T-cell and α_2HS-glycoprotein levels. These observations suggest that the cancer patients most likely to benefit therapeutically from adjuvant treatment with thymosin are those with relatively low initial T-cell levels and other parameters of cellular immunity.

[Cancer Treat Rep 62:1787–1790, 1978]

The demonstration that thymosin fraction 5 corrects cellular immune defects and improves the clinical status of children with thymic-dependent immune deficiencies (1) leads to the speculation that it may be beneficial in cancer patients because of the cellular immune defects associated with malignancies and the immunodepressive effects of conventional cancer therapies. We have explored these hypotheses in two experiments. The basis for the first experiment was the demonstration by Wara et al (1) that children with thymic deficiencies and low T-cell levels had marked increase in peripheral blood thymus-dependent lymphocyte (T-cell) levels in vitro after incubation with thymosin and that the clinical response to thymosin paralleled the results of this assay. We correlated the change in T-cell levels in vitro after incubation with thymosin with initial T-cell levels in patients with solid malignancies to determine if T-cell levels increased in vitro in patients with low T-cell levels. In the second experiment, parameters of cellular immunity prior to treatment were correlated with the clinical response to thymosin in vivo in patients with small cell carcinoma of the lung randomized to receive intensive chemotherapy in conjunction with thymosin or placebo. This group of patients was selected because of the marked impairment of cellular immunity in patients with small cell carcinoma of the lung (2) and the depression of in vitro parameters of cellular immunity associated with the chemotherapy regimen employed in this study.

MATERIALS AND METHODS

To assay the effect of thymosin in vitro on peripheral blood T-cell levels, we counted the percentage of lymphocytes forming spontaneous rosettes with sheep erythrocytes and determined the effects of the addition of thymosin on the percentage of rosettes in > 1500 blood specimens obtained from normal persons, untreated patients with solid malignancies, patients clinically cured of these malignancies, and patients receiving radiotherapy or chemo-

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[3]We are grateful to E. Trahan, A. Johnston, L. Paxton, D. White, and J. Olbrich for technical assistance, and H. Spiegel, Director, Laboratory of Clinical Biochemistry, Research Division, Hoffmann-La Roche, Inc, for determination of serum α_2HS-glycoprotein levels.

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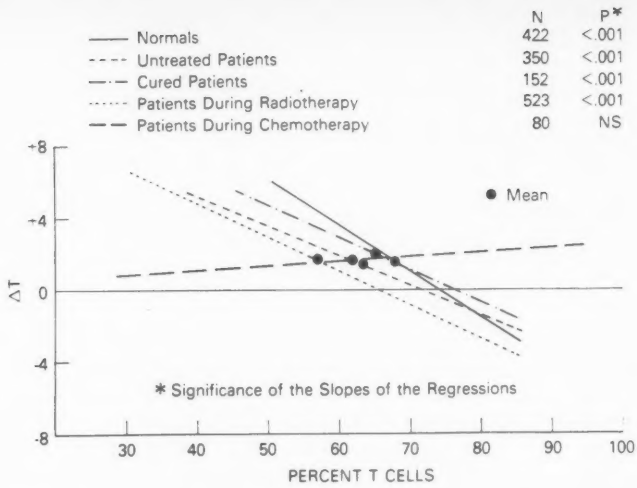

FIGURE 1.—Effect of thymosin in vitro on T-cell levels in peripheral blood specimens from normal persons and cancer patients: regression of change in initial T-cell levels after incubation of lymphocytes with thymosin adjusted for the regression toward the mean effect.

therapy for solid malignancies. The effect of thymosin was expressed as the difference between the percentage of lymphocytes forming spontaneous rosettes with sheep erythrocytes without thymosin and the percentage in specimens containing thymosin (3). The statistical significance of the correlation between the change in T-cell levels in response to thymosin in vitro and in the initial % T-cell level was evaluated using the Kendall-Stuart linear regression procedure (4) when both variables are measured with error. This regression procedure evaluates the strength of the correlation above what would be expected by chance from the regression toward the mean phenomenon. The error in the measurement in the percentage of T cells without thymosin was estimated from 100 sets of triplicate determinations.

To determine the relation between parameters of cellular immunity and response to thymosin in vivo we measured the pretreatment peripheral blood T-cell and serum α_2HS-glycoprotein (5,6) levels in patients receiving intensive chemotherapy for small cell carcinoma of the lung (7). In this trial, 55 patients who received chemotherapy were further randomized to receive either placebo, thymosin at 20 mg/m^2, or thymosin at 60 mg/m^2 twice weekly. The chemotherapy regimen during the first 6 weeks of therapy (without modification for hematologic toxicity) suppressed in vitro parameters of cellular immunity intensively compared to the subsequent chemotherapy regimens. Thymosin administration was limited to this initial phase of therapy. Response to chemotherapy and survival were determined and compared using classic life-table techniques (8–10).

RESULTS

The study of the effects of thymosin on peripheral blood T-cell levels in vitro showed a significant increase in the mean T cells after in vitro incubation with thymosin in normal persons and all cancer patients ($P < 0.001$–0.05). In addition, with the exception of patients receiving chemotherapy, there was a significant inverse relation between initial T-cell levels and T-cell levels after incubation with thymosin in vitro ($P < 0.001$). Specifically, the results showed a progressive increase in mean T-cell levels after incubation with thymosin among populations with low T-cell levels and progressively lower mean T-cell levels after incubation with thymosin in populations with high T-cell levels. Among patients receiving chemotherapy for a variety of solid tumors, T-cell levels increased after incubation with thymosin regardless of initial T-cell levels (fig 1) (3).

By life-table analysis of survival, the group of 21 patients who received chemotherapy and thymosin at a dose of 60 mg/m^2 had a significantly prolonged survival when compared to the 19 patients treated with chemotherapy and placebo ($P < 0.01$). The survival of 15 patients receiving chemotherapy and thymosin at a dose of 20 mg/m^2 did not differ

TABLE 1.—Relation of pretreatment total T cells and survival

Total T-cell level	Median survival in days*		Comparison between treatment groups by life-table analysis (Gehan's test, 1-sided)
	Chemotherapy and thymosin (60 mg/m²)	Chemotherapy alone	
High, > 775 cells/mm³	350 (9)	285 (11)	$P = 0.28$
Low, ≤ 775 cells/mm³	405 (12)	180 (8)	$P = 0.006$

*Nos. in parentheses = No. of patients.

TABLE 2.—Relation of pretreatment serum α_2HS-glycoprotein levels and survival

α_2HS-glycoprotein level	Median survival in days*		Comparison between treatment groups by life-table analysis (Gehan's test, 1-sided)
	Chemotherapy and thymosin (60 mg/m²)	Chemotherapy alone	
High, > 60.5 mg/dl	300 (10)	262 (11)	$P = 0.17$
Low, ≤ 60.5 mg/dl	495 (10)	180 (8)	$P = 0.039$

*Nos. in parentheses = No. of patients.

significantly from that of patients receiving chemotherapy alone ($P = 0.34$, one-sided) (7).

In the correlation between initial parameters of cellular immunity and clinical response to thymosin in vivo (11), the median T-cell level (775 cells/mm³) and serum α_2HS-glycoprotein level (60.5 mg/dl) for the entire population of 55 patients with small cell carcinoma of the lung were determined; pretreatment levels of each parameter above the median were scored as "high" and levels at or below the median were scored as "low." Comparison of the treatment groups demonstrated that patients with low pretreatment total T-cell or low pretreatment α_2HS-glycoprotein levels receiving chemotherapy and thymosin at 60 mg/m² achieved longer median survival than a similarly defined group of patients receiving chemotherapy only. For patients with high pretreatment total T-cell or high α_2HS-glycoprotein levels, the survival advantage among patients receiving thymosin at 60 mg/m² was not as pronounced (tables 1 and 2).

For those with either high pretreatment total T-cell or high α_2HS-glycoprotein levels, survival in patients treated with thymosin at 60 mg/m² was not significantly longer than in those receiving chemotherapy only ($P = 0.28$ and 0.17 respectively, one-sided). However, among the patients with either low pretreatment total T-cell or α_2HS-glycoprotein levels, those receiving thymosin at 60 mg/m² had significantly enhanced survival in comparison to patients receiving chemotherapy alone ($P = 0.006$ and 0.039 respectively, one-sided). Analysis of the data by tests for interaction, using the Cox life-table regression procedure (10), suggested that thymosin at 60 mg/m² differentially affected survival based on initial levels of total T cells ($P = 0.17$) and α_2HS-glycoprotein ($P < 0.05$) (11). Thus, it appears that the association between survival and type of treatment may depend upon pretreatment total T-cell or α_2 HS-glycoprotein levels.

DISCUSSION

In children with thymic deficiencies, the clinical response to thymosin (increase in cellular immunity and improvement in clinical status) was associated with an increase in T-cell levels toward the normal range but not to levels significantly above the normal range. The results of our in vitro studies provide an interesting parallel in that, in general, among populations with low T-cell levels, the levels increased in vitro after incubation with thymosin, but among those with high T-cell levels, the levels decreased after incubation with thymosin. Together, these data suggest that the primary immunologic effect of thymosin may be amelioration of cellular immune defects rather than induction of a hyperimmune state. The data parallel the findings in patients with small cell carcinoma of the lung: the correlations between pretreatment T-cell and α_2HS-glycoprotein levels and response to thymosin in vivo showed that among the patients receiving thymosin in addition to chemotherapy, the greatest increase

in survival occurred in those with either low pretreatment T-cell or α_2HS-glycoprotein levels, but among patients with either high pretreatment T-cell or α_2HS-glycoprotein levels, the survival of patients receiving chemotherapy and thymosin at 60 mg/m^2 was not significantly greater than in those receiving chemotherapy alone.

Despite the correlation between low pretreatment parameters of cellular immunity and clinical response to thymosin in the clinical trial, the mechanism by which thymosin increased survival cannot be discerned from this study. Pertinent here is that the response to chemotherapy (complete response, partial response, no response, or tumor progression) did not differ among the treatment groups. Specifically, the complete response rates among patients receiving chemotherapy and thymosin at 60 mg/m^2 (42.9%) and patients receiving only chemotherapy (47.4%) did not differ. Furthermore, significant differences in survival among patients receiving chemotherapy and thymosin at 60 mg/m^2 and those receiving chemotherapy alone were found only among patients achieving a complete response with chemotherapy. Thus, thymosin did not appear to significantly influence tumor response to chemotherapy, but among patients in whom there was complete clinical regression of tumor with chemotherapy, those receiving chemotherapy and thymosin at 60 mg/m^2 survived longer than those receiving chemotherapy alone (7). This unique manifestation of a therapeutic effect of thymosin remains unexplained; therefore, it is prudent to assume that beneficial effects occurring from the administration of thymosin in conjunction with conventional therapy may differ qualitatively with the therapies employed (eg, chemotherapy, radiotherapy, or surgery) and also with the type of tumor being treated. This conclusion emphasizes the need for meticulous monitoring of parameters of cellular immunity in all trials with thymosin as a preliminary step in discerning the clinical settings in which administration of thymosin will have adjuvant therapeutic effect in cancer therapy.

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Therapeutic Activity of Maleic Anhydride-Vinyl Ether Copolymers Against Spontaneous, Autochthonous Murine Mammary Tumors

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SUMMARY

We have tested a series of five maleic anhydride-vinyl ether copolymers of varying molecular weight for antitumor activity against spontaneous, autochthonous murine breast tumors (average initial tumor weight, approximately 200 mg). Four of the five compounds demonstrated statistically significant inhibition of tumor growth at nontoxic doses. (The fifth compound demonstrated antitumor activity only at toxic doses.) To our knowledge, this is the first report of significant activity in a spontaneous solid tumor system in a truly therapeutic setting (ie, with systemic administration only after the autochthonous tumors are clinically evident) with immunologic treatment alone.

[Cancer Treat Rep 62:1791–1796, 1978]

Pyran copolymers have yielded provocative antitumor activity in several transplantable murine tumor systems when administered alone, in conjunction with chemotherapy, or with a tumor cell vaccine (1–4). The antitumor activity is categorized as immunologic in nature, and it has been suggested that the antitumor activity of macrophages activated after administration of pyran may account for the beneficial effect (5–8).

The original pyran preparation contained a mixture of maleic anhydride-vinyl ether (MVE) copolymers of varying molecular weights. The undesirable level of toxicity associated with this material prompted the recent development of a synthetic process for the production of isolated molecular weight fractions in the hope that antitumor activity and host toxicity could be dissociated. Because of the potential therapeutic usefulness of these compounds, we have tested a series of five of these copolymers, varying in molecular weight from approximately 12,500 to 52,600, for in vivo antitumor activity in our model system of spontaneous, autochthonous breast cancer in (BALB/cf C3H × DBA/8)F_1 ($CD8F_1$) hybrid mice.

Although the usual, repeatedly transplanted murine tumor lines have been invaluable in the identification of presently available cancer chemotherapeutic drugs, it appears likely that a spontaneous animal tumor model will furnish more relevant information concerning immunologic agents for translation into the human clinical situation. The spontaneous mammary carcinoma in $CD8F_1$ mice provides such a model. Comparison of spontaneous mouse and human breast tumors reveals similarities of morphology, anatomic spreading, growth and progression through a precancerous stage, and genetic and hormonal factors (9). Murine mammary tumors are antigenic in their host of origin as are human breast cancers (10–13). Of pertinence to immunotherapy studies, synergism between host antitumor immunity, surgery, and combination chemotherapy has been demonstrated in the $CD8F_1$ tumor system (11,14). In addition, the $CD8F_1$ breast tumors have been shown to be sensitive to all eight agents tested that are considered unequivocally active against human breast cancer (15). The latter chemotherapeutic correlation augurs well for the appropriateness of this animal model for clinically predictive therapy studies, including immunotherapy studies.

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MATERIALS AND METHODS

Murine Tumor System (Spontaneous, Autochthonous Mammary Carcinomas)

$CD8F_1$ hybrid mice that were infected maternally with the mammary tumor virus were obtained from our colony which has been described previously (9,15). For each experiment, available mice bearing single spontaneous breast tumors arising during the preceding week were selected. On Day 0 (day of treatment), all tumors were measured and the mice were then distributed among experimental groups of 12–15 mice per group so that mice carrying tumors of approximately equal weight were represented in each group. The initial tumor size varied slightly from one experiment to another, but in general the average initial tumor weight was approximately 200 mg. Experimental groups were treated with a single ip administration of the appropriate MVE compound at one or more doses on Day 0. Controls received a single ip injection of the appropriate volume of phosphate-buffered saline (PBS); tumors were then measured at weekly intervals for 21 days.

Maleic Anhydride-Vinyl Ether Copolymers

The compounds were synthesized and supplied by Hercules, Inc, Wilmington, Del. They were all MVE 2:1 copolymers designated MVE-1 through MVE-5 with estimated molecular weights of 12,500, 15,500, 21,300, 32,000, and 52,600 respectively.

Immediately before use, each compound was suspended in PBS at pH 7.2 and the pH was adjusted to between 7.2 and 7.4 by dropwise addition of NaOH (0.3–2.0 N depending on the concentration of the MVE compound) on a magnetic stirrer. After stirring at room temperature for 60 minutes, the pH was checked again and any necessary adjustments were made. The solution was then brought to volume by the addition of PBS and administered ip so that the appropriate dose was contained in 0.1 ml/10 g of mouse body weight.

Tumor Measurements

The tumors were considered to be prolate ellipsoids with one long axis and two short axes assumed to be equal. Two axes of the tumor (the longest axis, L, and the shortest axis, W) were measured with the aid of a vernier caliper. Tumor weight was estimated according to the formula: tumor weight (mg) = L (mm) × [W (mm)^2]/2 (16). The Student's t test was used for statistical evaluation of differences between treated and control groups.

RESULTS

Each of the MVE copolymers (MVE-1 through MVE-5) was tested for therapeutic antitumor activity in two separate experiments and the results are presented in tables 1–5. A single ip administration of MVE-1 at doses of either 1000 mg/kg (experiment H3) or 500 mg/kg (experiment H12) produced statistically significant inhibition of tumor growth throughout the 21-day observation period (table 1). MVE-1 was well-tolerated at these doses and there were no drug-related deaths. Although we have also observed tumor inhibition after the administration of lower doses of MVE-1 (not shown), the magnitude of the activity usually was not great enough to be considered statistically significant.

Results obtained with MVE-2 (table 2) were very similar to those obtained with MVE-1. MVE-2 was well-tolerated with no toxic deaths when administered at doses of 1000 mg/kg (experiment H3) or 500 mg/kg (experiment H13). At a dose of 1000 mg/kg, statistically significant tumor inhibition was observed throughout the 21-day observation period. At a dose of 500 mg/kg, tumor inhibition was statistically significant on Days 7 and 14 although

TABLE 1.—Positive therapeutic activity of MVE-1 against spontaneous autochthonous $CD8F_1$ breast tumors*

		Day 7		Day 14		Day 21		
Experiment No.	Dose (mg/kg)	Tumor weight (mg)	% inhibition	Tumor weight (mg)	% inhibition	Tumor weight (mg)	% inhibition	% drug mortality
H3	1000	299	69†	733	68‡	1623	53§	0
	Control	960		2307		3447		
H12	500	266	40§	409	46§	846	52§	0
	Control	444		764		1761		

*Average tumor weight on the day of treatment (Day 0) was as follows: experiment H3 = 266 mg; experiment H12 = 162 mg.
†$P = < 0.001$ (statistical comparison to control).
‡$P = < 0.01$ (statistical comparison to control).
§$P = < 0.05$ (statistical comparison to control).

TABLE 2.—Positive therapeutic activity of MVE-2 against spontaneous autochthonous CD8F$_1$ breast tumors*

Experiment No.	Dose (mg/kg)	Day 7 Tumor weight (mg)	Day 7 % inhibition	Day 14 Tumor weight (mg)	Day 14 % inhibition	Day 21 Tumor weight (mg)	Day 21 % inhibition	% drug mortality
H3	1000	344	64†	720	69†	1137	67‡	0
	Control	960		2307		3447		
H13	500	192	59†	419	63†	1025	23	0
	Control	465		1144		1330		

*Average tumor weight on the day of treatment (Day 0) was as follows: experiment H3 = 266 mg; experiment H13 = 199 mg.
†$P = < 0.01$ (statistical comparison to control).
‡$P = < 0.05$ (statistical comparison to control).

TABLE 3.—Positive therapeutic activity of MVE-3 against spontaneous autochthonous CD8F$_1$ breast tumors*

Experiment No.	Dose (mg/kg)	Day 7 Tumor weight (mg)	Day 7 % inhibition	Day 14 Tumor weight (mg)	Day 14 % inhibition	Day 21 Tumor weight (mg)	Day 21 % inhibition	% drug mortality
H3	100	493	49†	1116	52‡	2114	39	0
	1000	346	64†	636	72†	1377	60	10
	Control	960		2307		3447		
H15	100	317	44‡	619	31	1484	20	0
	300	218	62†	324	64†	697	62	7
	Control	568		897		1848		

*Average tumor weight on the day of treatment (Day 0) was as follows: experiment H3 = 266 mg; experiment H15 = 175 mg.
†$P = < 0.01$ (statistical comparison to control).
‡$P = < 0.05$ (statistical comparison to control).

TABLE 4.—Lack of anticancer activity of MVE-4 at nontoxic doses against spontaneous autochthonous CD8F$_1$ breast tumors*

Experiment No.	Dose (mg/kg)	Day 7 Tumor weight (mg)	Day 7 % inhibition	Day 14 Tumor weight (mg)	Day 14 % inhibition	Day 21 Tumor weight (mg)	Day 21 % inhibition	% drug mortality
H3	100	537	44†	855	63†	1475	57†	10
	1000	214	78‡	654	72†	1251	64†	40
	Control	960		2307		3447		
H16	25	331	26	727	5	1200	13	0
	50	309	31	598	22	1011	27	7
	100	213	52†	516	33	969	30	47
	Control	445		766		1387		

*Average tumor weight on the day of treatment (Day 0) was as follows: experiment H3 = 266 mg; experiment H16 = 187 mg.
†$P = < 0.05$ (statistical comparison to control).
‡$P = < 0.01$ (statistical comparison to control).

TABLE 5.—Positive therapeutic activity of MVE-5 against spontaneous autochthonous $CD8F_1$ breast tumors*

Experiment No.	Dose (mg/kg)	Day 7 Tumor weight (mg)	Day 7 % inhibition	Day 14 Tumor weight (mg)	Day 14 % inhibition	Day 21 Tumor weight (mg)	Day 21 % inhibition	% drug mortality
H3	100	330	66†	555	76‡	1284	63†	30
	1000	—	—	—	—	—	—	90
	Control	960		2307		3447		
H10	8	620	17	920	19	1569	23	0
	25	493	34	462	59†	814	60‡	0
	75	438	41	380	67‡	745	64‡	33
	Control	745		1135		2042		

*Average tumor weight on the day of treatment (Day 0) was as follows: experiment H3 = 266 mg; experiment H10 = 273 mg.
†$P = < 0.05$ (statistical comparison to control).
‡$P = < 0.01$ (statistical comparison to control).

the antitumor activity was not significant by 21 days after treatment. Again, as with MVE-1, the magnitude of tumor inhibition resulting from the administration of lower doses (data not shown) usually was not great enough to be considered statistically significant.

Administration of MVE-3 at 1000 mg/kg also resulted in statistically significant inhibition of tumor growth (table 3), however, a mortality rate of 10% was observed at this dose. In a separate experiment (H15, table 3), a dose of 300 mg/kg also produced statistically significant antitumor activity, but again a mortality rate of 7% was observed. With a dose of 100 mg/kg (experiments H3 and H15), we observed statistically significant tumor inhibition with no drug-associated mortality. At present, we do not know whether or not this compound will have any therapeutic activity at lower doses in this spontaneous $CD8F_1$ breast tumor system.

MVE-4 also showed significant antitumor activity at a dose of 1000 mg/kg (table 4). However, within the first week after administration of this dose a mortality rate of 40% was observed. A dose of 100 mg/kg also produced significant tumor inhibition, but drug mortality rates of 10% and 47% were seen at this dose in experiments H3 and H16 respectively. Drug toxicity was not observed at a dose of 25 mg/kg, but at this dose tumor growth was essentially unaffected. Even at a dose as low as 50 mg/kg, a drug mortality rate of 7% was observed, but antitumor activity was not significant. These results indicate that MVE-4 is only active against spontaneous tumors at toxic doses and, therefore, must be considered ineffective as a therapeutic agent.

MVE-5 was extremely toxic at a dose of 1000 mg/kg, and within 1 week after a single ip administration at this dose 90% of the mice were dead. At a dose of 100 mg/kg, MVE-5 produced statistically significant tumor growth inhibition throughout the 21-day observation period; however, this dose also produced an intolerable mortality rate of 30% (experiment H3, table 5). In a second experiment (experiment H10, table 5), significant antitumor activity resulting from administration of 75 mg/kg was accompanied by a drug-associated mortality rate of 33%, whereas a dose of 25 mg/kg yielded essentially similar tumor inhibition with no toxic deaths. At a lower dose of 8 mg/kg, MVE-5 essentially showed no effect on tumor growth. Although the therapeutic index of MVE-5 may be small, this compound appears to be useful at a dose of 25 mg/kg.

For a comparative view of the effects of each of the five MVE copolymers on the tumor and on the host, we have tabulated results obtained in a single experiment where each of the compounds was administered at a dose of 1000 mg/kg (table 6). Antitumor activity is presented in terms of percent inhibition of tumor growth (in comparison to diluent-treated control) at 14 days after administration of the compounds. Percent drug mortality, observed within the first week after administration, remained unchanged throughout the 21-day observation period. At this dose (1000 mg/kg), all of the compounds with the exception of MVE-5 (which was too toxic for evaluation) produced a significant and similar degree of tumor inhibition (68%–72%). However, host toxicity varied greatly (from 0 to 90% mortality) and was directly proportional to the molecular weight of the compounds. MVE-1 and MVE-2 were well-tolerated with no lethal toxicity. Although tolerated doses with significant antitumor activity were found for MVE-3 and MVE-5 (100 and 25 mg/kg respectively) as indicated previously, MVE-4 was always toxic at effective doses. It is obvious, therefore, that the therapeutic usefulness of a mixture of

TABLE 6.—Comparative effects of 5 MVE copolymers within a single experiment in spontaneous tumor-bearing $CD8F_1$ mice

Compound	Approximate molecular weight	Dose (mg/kg)	% tumor inhibition*	% drug mortality
MVE-1	12,500	1000	68†	0
MVE-2	15,500	1000	69†	0
MVE-3	21,300	1000	72†	10
MVE-4	32,000	1000	72‡	40
MVE-5	52,600	1000	—	90

*Tumor inhibition was measured 14 days after a single ip administration of the MVE copolymer.
†$P = < 0.01$ (statistical comparison to control).
‡$P = < 0.05$ (statistical comparison to control).

these copolymers (as in the original pyran preparation) would be limited by the toxicity of the higher molecular weight components.

DISCUSSION

The data clearly indicate that four of the five MVE copolymers tested (MVE-1, -2, -3, and -5) demonstrate significant antitumor activity without apparent host toxicity when tested against spontaneous, autochthonous $CD8F_1$ breast tumors. (MVE-4 was an exception in that significant antitumor activity was obtained only at toxic doses.) To our knowledge, this is the first report of significant activity in a spontaneous solid tumor system in a truly therapeutic setting (ie, with systemic administration only after the autochthonous tumors are clinically evident) with immunologic treatment alone.

As indicated above, earlier preparations of pyran containing a mixture of varying molecular weight copolymers were found to be toxic when clinically evaluated. The advantage of the individual molecular weight fractions of the pyran copolymers is apparent from the comparative results shown in table 6. Host toxicity was directly related to the molecular weight of the copolymer. In a mixture of these compounds, toxicity associated with the higher molecular weight components such as MVE-4 and MVE-5 would obviously be a limiting factor. With the individual compounds, safe and effective doses have been found for each, with the exception of MVE-4. It is expected that the ability to utilize the individual compounds at safe and effective doses will be particularly important in future studies of combined modality therapy where additive or synergistic toxicity may be anticipated as a problem when these immunologic agents are used in conjunction with surgery and/or chemotherapy.

The fact that the MVE copolymers were effective here in a truly therapeutic situation (ie, in an autochthonous situation where tumors are clinically evident), as opposed to the usual prophylactic or early post-threshold challenge protocols used in common transplantable murine tumor systems, is particularly relevant for translation of results to the human disease. The net result of immunologic manipulation in any tumor host system is dependent upon the interaction of the particular therapeutic maneuver with a particular tumor in a particular host and with the given tumor-associated immune situation at a particular time. In the spontaneous $CD8F_1$ breast tumor system used here, therapy is initiated in a naturally and slowly evolved, full-blown, and obviously unsuccessful immune setting. It is precisely this type of immune setting which must be manipulated in the therapy for human disease.

Finally, however, it must be understood that we are not providing any support for the proposed immunomodulating mechanism of action of these pyran copolymers. Neither are we providing any contradictory evidence. Rather, we are working under the hypothesis suggested by others (4–8) that the antitumor activity associated with these drugs is an indirect manifestation of their effect on immune systems. It will be an important feature of future study with these compounds in our spontaneous $CD8F_1$ tumor system to determine whether or not this hypothesis is correct.

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Relationship of Molecular Weight to Antiviral and Antitumor Activities and Toxic Effects of Maleic Anhydride-Divinyl Ether (MVE) Polyanions

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SUMMARY

The molecular weight (MW) and dose dependency of several of the toxic effects and antitumor and antiviral activities of a new series of five maleic anhydride-divinyl ether copolymers (MVE) were established. Each polyanion preparation was relatively homogeneous and exhibited a narrow MW range, from 12,500 (MVE-1) to > 52,000 (MVE-5). All of the polyanions were effective as adjuvants to surgery against the metastatic Lewis lung carcinoma, and also exhibited marked antitumor activity against the P815 mastocytoma. MVE-1 retained antitumor activity while losing considerable antiviral activity. This polyanion also exhibited the least toxicity with regard to criteria such as sensitization to the lethal effects of endotoxin, inhibition of reticuloendothelial function, and depression of the microsomal mixed functional oxidase system. The MVE-4 (MW, 32,000) and MVE-5 (MW, 52,600) polyanions exhibited potent antitumor and antiviral activity, but also demonstrated dose-dependent toxic effects.

[Cancer Treat Rep 62:1797–1805, 1978]

The maleic anhydride-divinyl ether copolymer polyanions (MVE) have previously been demonstrated to induce marked host resistance to a variety of virus infections (1–5) and leukemic and solid tumors (6–11), as well as exhibiting immunomodulatory activity (1,12,13). The mechanisms of antiviral and antitumor action appear to be closely related to the ability of the compounds to activate macrophages for antitumor and antiviral activity (5,7,8,10,14). The clinical usefulness of this class of drugs has been limited by toxic effects that appear to be inherent in poorly degradable large molecular weight (MW) polyanions (15–17). A series of MVE compounds was synthesized with MW ranging from 12,000 to > 52,000. Each preparation was relatively homogeneous, exhibiting a narrow MW range. The present results describe the MW dependency of several of the toxic effects and antiviral and antitumor activities of this new series of compounds.

MATERIALS AND METHODS

Mice.—BALB/c mice were used for the antiviral studies, C57BL/6J mice for the studies with the Lewis lung carcinoma, and CDF_1 mice for the studies with the P815 mastocytoma and toxicologic studies. All mice were acclimated for at least 1 week prior to experimental use.

Tumors.—The Lewis lung carcinoma was maintained by im passage of 10^6 tumor cells every 3 weeks in C57BL/6J mice (11). The P815 mastocytoma was maintained by weekly passage of 1×10^5 cells in the ascites form in DBA/2 mice.

Virus challenge.—BALB/c mice were challenged iv with ten LD50 of encephalomyocarditis (EMC) virus, ATCC VR129, prepared as previously described (1).

Drugs.—MVE compounds and pyran copolymer (NSC-46015, XA 124-177) were received from Dr. D. S. Breslow, Hercules, Inc (Wilmington, Del). The compounds were dissolved in phosphate buffered

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[3]We wish to acknowledge the excellent technical assistance of Barbara Kipps, Patty Hallett, and Ann Munson for antitumor and antiviral studies, and Beverly Barrett and Mary Stevenson for toxicologic studies.

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saline (PBS) at room temperature with stirring, and the pH was adjusted to 7.0–7.4. Drug solutions were stored at 4°C for ≤ 1 week. Cyclophosphamide (CPS) and methyl-CCNU (MCCNU) were supplied by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md). MCCNU was dissolved in ethyl alcohol, emulphor, and 0.15 M NaCl (1:1:8). CPS was dissolved in PBS, the solutions were kept on ice, and the drugs were inoculated within 30 minutes of preparation. All drug doses were prepared for inoculation of 0.01 ml/g of body weight.

Macrophage antitumor assay.—Peritoneal exudate cells were removed from mice that had been inoculated ip 7 days previously with PBS, pyran copolymer, or the MVE compounds (25 mg/kg). The percent of macrophages was determined by the presence of nonspecific esterase activity. The percent of cytotoxicity against Lewis lung carcinoma or secondary mouse embryo fibroblasts was determined in macrophage target-cell combinations cultured in Lab-Tek chambers as previously described, using 10:1 and 5:1 peritoneal cell:target cell ratios (10).

Acute toxicity.—Groups of seven to ten CDF_1 female mice were inoculated iv with the MVE polyanions in four to six doses between 75 and 150 mg/kg. The drug dose was administered in 0.01 ml/g at a constant rate. The death rate was recorded over a 14-day period and the LD50 and slope were calculated.

Sensitization to bacterial endotoxin (1, 21).—Groups of ten C57BL/6J male mice were injected iv with 25 mg/kg of MVE polyanions, followed 24 hours later by iv administration of various doses of *Escherichia coli* lipopolysaccharide (LPS) (Difco, 011:B4 Westphal preparation). The death rate was observed for 14 days, and the LD50 was calculated.

Hematologic parameters.—CDF_1 female mice were injected iv with 25 mg/kg of MVE polyanions. Twenty-four hours later, blood was collected by cardiac puncture into 3.8% sodium citrate. The hemoglobin level was determined by cyanomethemoglobin, and leukocytes, erythrocytes, and platelets were enumerated on a Coulter counter model ZB-I.

Hepatic microsomal mixed functional oxidase (MMFO) enzymes.—Twenty-four hours after iv injection of MVE polyanions, CDF_1 mice were killed by cervical dislocation, and the livers were removed, weighed, rinsed, and homogenized at 0°C–4°C in 4 volumes of 1.15% KCl/0.01 M KH_2PO_4-K_2HPO_4 buffer at pH 7.4. The homogenate was centrifuged at 9000 × *g* for 10 minutes, and the resulting supernatant fluid was re-centrifuged at 100,000 × *g* for 1 hour. Microsomes were resuspended in 0.05 M phosphate buffer, pH 7.4 (0.5 g of liver/ml), and aliquots were taken for immediate study. Microsomal cytochrome P-450 content was determined at room temperature in dithionite-reduced microsomes by measuring the difference in absorbance (450–490 nm) and using 91 $cm^{-1}nm^{-1}$ as the extinction coefficient. Microsomal aminopyrine *N*-demethylase activity was determined by the measurement of formaldehyde formation and aniline hydroxylase activity by the measurement of *p*-aminophenol formation (18).

Activity of the reticuloendothelial system (RES).—The functional activity of the RES was assessed as previously described (19). CDF_1 female mice were injected iv with MVE polyanions 24 hours prior to measurement of the vascular clearance rate and organ uptake of ^{51}Cr sheep erythrocytes (SRBC) injected iv in a dose of 5×10^8 cells/0.01 g of body weight.

Statistics.—The chi square with Yate's correction factor was used for analysis of mortality data. The Student's t test or analysis of variance with the Dunnett t test was used to compare experimental groups to the control. The method of Litchfield and Wilcoxon (20) was used to determine the LD50 and slopes of dose-response curves.

RESULTS

Relationship of MW to various toxicologic parameters.—The acute toxicity of MVE polymers increased with increasing MW; MVE-1 was significantly less toxic than MVE-2, -3, -4, and -5, and MVE-2, -3, and -4 were significantly less toxic than MVE-5 (table 1). Lethality occurred within 3 minutes of injection and the cause of death appeared to be an effect on the cardiovascular and/or respiratory system(s). Necropsy at the time of death showed no macroscopic pathology. MVE-3, -4, and -5 also increased the susceptibility of mice to the lethal effects of LPS by 30–40-fold and MVE-2 by threefold, while MVE-1 did not sensitize mice to LPS (table 1). The MVE polymers did not markedly affect hemoglobin or erythrocytes 24 hours after a 25-mg/kg injection (table 2). MVE-5 caused a twofold increase in peripheral blood leukocytes, which was due to an increase in the granulocytic and monocytic leukocytes and which returned to control values in 7 days. The most significant hematologic change was related to the thrombocytopenia of as much as 40% caused by MVE-3, -4, and -5, and which returned to control values in 5 days. The MVE compounds affected several parameters of the hepatic MMFO system (table 3). The MVE polymers did not significantly decrease microsomal protein, although slight decreases occurred after treatment with the higher MW copolymers. When expressed on the basis of

TABLE 1.—Relationship between MW of MVE polymers and acute toxicity and sensitivity to bacterial endotoxin

Polymer	MW†	LD50* (95% confidence limits)	Slope (95% confidence limits)	LD50 of bacterial endotoxin‡ (mg/kg)
NSC-46015		78 (73–83)	1.112 (1.106–1.118)	0.3
MVE-1	12,500	112 (103–122)	1.103 (1.100–1.106)	24.0
MVE-2	15,500	98 (94–102)	1.047 (1.046–1.047)	7.0
MVE-3	21,300	94 (89–99)	1.061 (1.060–1.061)	0.8
MVE-4	32,000	95 (91–98)	1.040 (1.040–1.041)	0.4
MVE-5	52,600	86 (81–91)	1.120 (1.117–1.123)	0.5
Control (0.15 M NaCl)	—	—	—	21.0

*CDF_1 female mice were administered MVE polymers iv in various doses in 0.01 ml/g of body weight. Mice were observed for death for 14 days. LD50 and slope determinations were calculated by the method of Litchfield and Wilcoxon (ref 20).

†MW was determined by gel permeation chromatography.

‡C57BL/6J male mice were administered 25 mg/kg of the polymers followed 24 hrs later by various doses of *E. coli* endotoxin. Deaths were recorded over 14 days.

TABLE 2.—Effect of MVE polymers on hematologic parameters*

Polymer	Hemoglobin (g/100 ml)	Erythrocytes ($\times 10^6$/mm^3)	Leukocytes ($\times 10^3$/mm^3)	Platelets ($\times 10^5$/mm^3)
Control (0.15 M NaCl)	19.3 ± 0.3	8.09 ± 0.5	9.0 ± 0.3	2.72 ± 0.1
MVE-1	19.7 ± 0.3	9.04 ± 0.2	10.7 ± 1.3	2.75 ± 0.2
MVE-2	19.7 ± 0.3	9.49 ± 0.2†	10.9 ± 1.1	2.97 ± 0.1
MVE-3	20.9 ± 0.6	9.15 ± 0.1	9.0 ± 0.9	1.66 ± 0.1†
MVE-4	18.4 ± 0.8	9.23 ± 0.2	8.6 ± 1.4	1.41 ± 0.1†
MVE-5	19.3 ± 0.9	8.96 ± 0.1	18.2 ± 2.3†	1.47 ± 0.1†

*CDF_1 female mice were injected with 25 mg/kg of MVE polymers iv and cardiac blood was taken in 3.8% sodium citrate. Hemoglobin was determined by cyanomethemoglobin and cell counts were determined on a Coulter counter.

†$P < 0.05$.

microsomal protein, cytochrome P-450 content was significantly lowered after treatment with either NSC-46015, MVE-2, -3 -4, or -5. Similar decreases were observed in the *N*-demethylation of aminopyrine, while there were greater decreases in biotransformation of aniline. When calculated on the basis of liver weight or per total animal, the reduction of each of the parameters studied was even greater. In general, the reduction of each of the MMFO parameters measured was directly related to the MW of the polymers. All of the MMFO parameters returned to control levels between 7 and 14 days after treatment. The MVE polymers also altered the functional activity of the RES 24 hours after iv injection of 25 mg/kg, producing MW-dependent effects on the vascular clearance rate and hepatic uptake of ^{51}Cr SRBC (table 4). MVE-1, -2, and -3 produced an enhancement of hepatic phagocytosis and vascular clearance rate of SRBC by Day 3 which remained for at least 7 days (data not shown). MVE-4 and -5 produced a depression of hepatic phagocytosis and vascular clearance rate

TABLE 3.—Effect of MVE polymers on microsomal protein, cytochrome P-450 content, aminopyrine demethylase, and aniline hydroxylase*

Polymer	Microsomal protein†	Cytochrome P-450 content‡	Aminopyrine *N*-demethylase§	Aniline hydroxylase¶
Control (0.15 M NaCl)	18.4 ± 1.0	0.808 ± 0.035	7.50 ± 0.36	1.66 ± 0.06
NSC-46015	16.4 ± 0.9	0.487 ± 0.040**	4.57 ± 0.45**	0.88 ± 0.11**
MVE-1	18.0 ± 0.5	0.676 ± 0.051	6.47 ± 0.28	1.32 ± 0.10
MVE-2	17.6 ± 0.3	0.619 ± 0.064**	5.86 ± 0.54**	1.24 ± 0.11**
MVE-3	17.9 ± 0.6	0.545 ± 0.054**	5.27 ± 0.46**	0.94 ± 0.11**
MVE-4	16.9 ± 1.1	0.437 ± 0.019**	4.48 ± 0.56**	0.76 ± 0.05**
MVE-5	15.8 ± 0.9	0.438 ± 0.049**	3.98 ± 0.25**	0.73 ± 0.06**

*CDF_1 female mice were administered MVE polymers iv in a dose of 25 mg/kg; 24 hrs later, the livers were excised and microsomes were prepared as indicated in Methods section (mean ± SE derived from at least 4 experiments).
†mg/g of liver.
‡nmols/mg of protein.
§nmols of formaldehyde formed/mg/min.
¶nmols of para-aminophenol/mg/min.
**$P < 0.05$ as compared to control.

TABLE 4.—Relationship of MVE polymer MW to functional status of the RES*

		Liver		Spleen		Lungs		Thymus
Polymer	Phagocytic index	Weight†	% uptake‡	Weight	% uptake	Weight	% uptake	Weight
Control (0.15 M NaCl)	0.073 ± 0.009	4.53 ± 0.09	73.3 ± 1.4	0.40 ± 0.03	7.0 ± 0.4	1.15 ± 0.12	0.39 ± 0.07	0.18 ± 0.01
MVE-1	0.069 ± 0.008§	4.96 ± 0.03	71.2 ± 0.18	0.44 ± 0.02	4.3§ ± 0.6	1.03 ± 0.11	0.27§ ± 0.03	0.15 ± 0.01
MVE-2	0.059 ± 0.016§	4.89 ± 0.14	60.8§ ± 3.2	0.55§ ± 0.02	8.6 ± 1.1	1.13 ± 0.15	0.52 ± 0.10	0.21 ± 0.01
MVE-3	0.029 ± 0.003§	5.66§ ± 0.11	20.6§ ± 4.4	0.58§ ± 0.03	29.7§ ± 2.8	0.94 ± 0.04	2.22§ ± 0.16	0.16§ ± 0.02
MVE-4	0.016 ± 0.004§	5.77§ ± 0.15	4.9§ ± 1.3	0.60§ ± 0.02	29.2§ ± 3.0	1.00 ± 0.07	4.08§ ± 0.70	0.11§ ± 0.02
MVE-5	0.015 ± 0.002§	5.90§ ± 0.11	4.5§ ± 0.9	0.66§ ± 0.04	18.6§ ± 3.6	1.09 ± 0.07	4.59§ ± 0.42	0.08§ ± 0.01

*Groups of CDF_1 female mice were administered MVE polymers in a dose of 25 mg/kg iv; 24 hrs later the functional status of the RES was assessed.
†Organ weight expressed as % of body weight.
‡% of injected ^{51}Cr SRBC/total organ.
§$P < 0.05$.

TABLE 5.—Effect of MW on antiviral activity of MVE polyanions against EMC virus*

Drug	% mortality at doses (mg/kg) of—						
	75	50	25	12.5	6.25	3.12	0
PBS							86
MVE-1	29†	44	80	50			
MVE-2	11†	11†	0†	40†			
MVE-3		0†	0†	0†	30†		
MVE-4			0†	0†	10†	30†	
MVE-5			0†	0†	0†	0†	
NSC-46015			0†	0†	0†	10†	

*BALB/c mice were inoculated iv with the drugs and challenged iv 24 hrs later with virus. Experimental groups included 7–10 mice, and the PBS control group included 15 mice.
†$P < 0.05$.

which returned to normal within 7 days. Splenic and lung uptake of SRBC was increased markedly in mice treated with MVE-3, -4, and -5. Hepatosplenomegaly and thymic atrophy occurred and were also MW-dependent. Thymic weight returned to normal by Day 7, but the increases in liver and spleen weight required at least 14 days to return to control levels (data not shown).

Relationship of MW to antiviral activity against EMC virus.—A clear MW- and dose-dependent increase in antiviral activity was observed with prophylactic treatment of mice with the MVE compounds 24 hours prior to iv virus challenge (table 5). The lowest MW compound, MVE-1, lost antiviral activity at doses < 75 mg/kg. MVE-2 was active at doses as low as 12.5 mg/kg while MVE-3 retained activity at 6.25 mg/kg. The two largest MW compounds, MVE-4 and -5, retained activity at 3.12 mg/kg. Both the largest MW compound (MVE-5) and the polydisperse polymer (NSC-46015) provided almost solid antiviral protection at 3.12 mg/kg.

Macrophage tumor-selective cytotoxicity.—Ip injection of the MVE compounds caused in 7 days a slight increase in the number of peritoneal cells and the proportion of esterase-positive macrophages (table 6). The adherent peritoneal cells from all groups of treated mice exhibited tumor-selective cytotoxicity for Lewis lung tumor cells with no toxicity for mouse embryo fibroblasts.

Relationship of MW to antitumor activity in combination surgery-MVE treatment against the Lewis lung carcinoma.—When mice bearing the Lewis lung carcinoma in the footpad were treated iv with MVE compounds beginning on Day 17 after tumor inoculation, no marked beneficial effect was observed (table 7). Similarly, when mice were treated by removing the primary tumor on Day 14, a time when the tumor had already metastasized to the lung, no increase in median survival time (MST) was afforded. However, when surgery was combined with MVE treatment, a significant prolongation in MST occurred. This effect was MW- and dose-dependent. With the small MW compound (MVE-2), only treatment at the high dose level (25 mg/kg) prolonged the MST. With MVE-4 both the middle (10 mg/kg) and high doses prolonged MST. All three doses of MVE-5 produced a significant prolongation of MST, and produced the greatest increased lifespan (ILS) of 138%.

Relationship of MW to antitumor effect of MVE compounds against the P815 mastocytoma footpad tumor.—In contrast to the Lewis lung carcinoma which metastasizes primarily to the lung, the P815

TABLE 6.—Macrophage antitumor activity*

Drug	No. of peritoneal exudate cells/mouse ($\times 10^6$)	% esterase	% cytotoxicity	
			Lewis lung	Mouse embryo fibroblasts
PBS	5.0	29	0	0
MVE-1	6.5	38	100	0
MVE-2	7.0	45	94	0
MVE-3	9.5	33	94	0
MVE-4	6.8	36	81	0
MVE-5	9.8	37	88	0
NSC-46015	11.0	44	88	0

*Peritoneal exudate cells were removed and tested 7 days after ip injection of drugs at 25 mg/kg for cytotoxicity against Lewis lung and secondary mouse embryo fibroblast cells.

TABLE 7.—Combination surgery and iv MVE treatment against Lewis lung carcinoma*

Treatment		MST ± SE in—	
Drug	Dose (mg/kg)	Lewis lung without surgery	Lewis lung with surgery
PBS	—	31.3 ± 0.9	29.9 ± 1.2
MVE-2	5	32.9 ± 3.0	28.2 ± 1.2
	10	31.0 ± 3.5	30.2 ± 1.8
	25	30.3 ± 2.0	37.8 ± 2.5†
MVE-4	5	26.4 ± 2.0†	33.3 ± 2.6
	10	31.3 ± 2.8	35.9 ± 1.5†
	25	33.8 ± 2.5	39.7 ± 2.5†
MVE-5	5	27.3 ± 2.1	39.2 ± 2.7†
	10	36.2 ± 2.2†	41.2 ± 2.1†
	25	29.9 ± 2.2	38.1 ± 1.8†

*C57BL/6J mice inoculated with tumor cells in the footpad (the leg was amputated or not by Day 14) and PBS or MVE was inoculated at the indicated doses on Days 17, 20, 24, 31, 34, and 37.

†$P < 0.05$.

mastocytoma metastasizes primarily to the liver and spleen (unpublished observations). Because the major target organs for MVE action are the liver and spleen (table 4), it was of interest to determine the effect of the MVE polyanions against this tumor. Mice were treated iv once a week for 6 weeks, beginning 7 days after tumor inoculation. The P815 mastocytoma footpad tumor metastasizes in 60% of mice by Day 10 and in 100% of mice by Day 15 (unpublished observations). Treatment with any of the MVE compounds significantly increased the MST of mice (table 8). However, the three largest MW MVE compounds were more effective than were MVE-1 and -2. The ILS produced by MVE-3, -4, and -5 was equivalent to that produced by the same dose of CPS. Moreover, a few long-term survivors were produced by either MVE or CPS treatment.

Lack of effect of MCCNU or of MCCNU-MVE combination against the Lewis lung carcinoma in C57BL/6J mice.—MCCNU, instead of surgery, was used as the treatment to reduce the primary tumor burden; treatment with MCCNU on Days 9 and 16 has proved effective in increasing the MST of BDF_1 mice bearing the sc Lewis lung carcinoma (9,22). However, treatment with MCCNU of C57BL/6J mice bearing either the footpad or im Lewis lung carcinoma did not significantly prolong the MST (table 9), although tumor growth was slightly inhibited (data not shown). The minimal effectiveness may be due to the increased toxicity of MCCNU for C57BL/6J as compared with BDF_1 mice (23). The combination of MVE treatment simultaneously with the first MCCNU injection did not increase the antitumor effectiveness of MCCNU (table 10). Indeed, the MST of several groups was slightly, but significantly, reduced when both MCCNU and MVE compounds were administered. Similar results were obtained when MVE treatment was delayed until Day 21, 5 days after completion of the MCCNU treatment.

Lack of effect of MVE in combination with local irradiation of the Lewis lung carcinoma.—^{60}Co irradiation, instead of surgery, was used as the treatment to reduce the primary Lewis lung footpad tumor; local irradiation of tumors with 2500, 3500, or 4500 rads significantly prolonged the MST of

TABLE 8.—Antitumor activity of MVE polyanions against P815 mastocytoma footpad tumor*

Treatment		MST ± SE† (days)	% ILS	Survivors on Day 100/ total treated
Drug	Dose (mg/kg)			
PBS	—	29.5 ± 0.9		0/8
MVE-1	25	37.3 ± 2.3	126	1/7
MVE-2	25	37.3 ± 2.1	126	0/8
MVE-3	25	52.8 ± 4.1	179	0/8
MVE-4	25	52.7 ± 4.8	179	3/8
MVE-5	25	47.8 ± 4.0	162	0/8
CPS	25	47.6 ± 1.1	161	0/8
CPS	50	65.0 ± 1.8	220	0/7
CPS	75	72.2 ± 1.5	245	2/8

*$CD2F_1$ mice were inoculated in the footpad with 5×10^5 P815 cells. MVE polymers, CPS, or PBS was injected iv once a wk for 6 wks beginning 7 days after tumor inoculation.

†All MSTs are significantly increased from the control.

TABLE 9.—Dose response of MCCNU against Lewis lung carcinoma*

Experiment No.	Drug (dose and route)	MST ± SE by route	
		Im	Footpad
1	Vehicle	24.8 ± 0.9	30.8 ± 2.9
	MCCNU (20 ip, 10 ip)	21.8 ± 1.3†	28.0 ± 2.8
	MCCNU (40 ip, 10 ip)	17.1 ± 1.4†	18.3 ± 0.8†
2	None	29.2 ± 1.6	35.3 ± 3.3
	Vehicle	29.9 ± 1.4	32.7 ± 2.8
	MCCNU (10 ip, 10 ip)	30.8 ± 1.0	36.3 ± 4.0
	MCCNU (20 ip, 10 ip)	29.6 ± 3.8	36.6 ± 3.3
	CPS (100 ip, 100 iv)	32.1 ± 1.4	36.0 ± 6.8

*C57BL/6J mice were inoculated with 3×10^5 Lewis lung cells im or in the footpad, and were treated with the indicated drug doses (mg/kg) by the indicated routes on Days 9 and 16.
†$P < 0.05$.

mice (table 11). The combination of MVE-3 with the ^{60}Co treatment did not increase the antitumor effect over that observed with radiation alone. Results with MVE-2, -4, and -5 were generally similar (data not shown).

DISCUSSION

The present results demonstrate MW- and dose-dependent relationships for a series of carefully characterized MVE polymers with regard to toxicologic and antitumor and antiviral activities. The smallest polyanion (MW, 12,500) retained antitumor activity while losing considerable antiviral activity.

TABLE 10.—Simultaneous MCCNU-MVE treatment*

Treatment		MST ± SE of mice treated with MVE and—	
Drug	Dose (mg/kg)	PBS	MCCNU
PBS	—	28.9 ± 0.3	27.0 ± 0.5
MVE-1	5	27.5 ± 1.2	24.1 ± 0.2†
	10	30.4 ± 1.6	24.4 ± 1.5†
	25	29.1 ± 1.2	27.9 ± 0.4
MVE-2	5	28.1 ± 0.6	28.2 ± 1.1
	10	29.6 ± 0.5	28.2 ± 0.2
	25	29.5 ± 0.3	30.2 ± 0.4
MVE-3	5	33.4 ± 0.4†	26.8 ± 0.6
	10	30.0 ± 0.7	29.9 ± 0.4
	25	28.9 ± 1.6	23.9 ± 1.0†
MVE-4	5	27.8 ± 0.4	28.0 ± 0.3
	10	33.5 ± 0.4†	26.1 ± 0.7
	25	30.1 ± 0.9	23.2 ± 0.2†
MVE-5	5	27.0 ± 0.9	25.8 ± 0.9
	10	29.8 ± 0.2	28.9 ± 0.9
	25	28.5 ± 0.4	28.8 ± 0.4

*C57BL/6J mice were inoculated im with 3×10^5 Lewis lung cells and treated ip on Days 9 and 16 with MCCNU (10 mg/kg) and ip on Day 9 with MVE.
†$P < 0.05$.

Our previous results with low MW polyanions of similar chemistry demonstrated that polyanions with an MW of < 14,900 exerted no antiviral activity at 25 mg/kg against EMC virus (17). The present results demonstrate that antiviral activity can be exhibited with a compound with an MW of 12,500, but that considerably more drug (75 mg/kg) must be administered or the dose of the virus challenge must be decreased.

All of the MVE compounds activated macrophages for tumor-selective cytotoxicity in vitro and for antitumor activity in vivo. These results are similar to our data with other types of polyanionic compounds, in which we demonstrated that compounds that activated macrophages for antitumor cytotoxicity in vitro also exhibited antitumor activity in vivo (10). Similarly, low MW polyanions (MW, 5500–8000) that did not activate macrophages did not exhibit antitumor activity in vivo. The present results have shown that the larger (MW, 21,300–52,000) MVE compounds exhibit more antitumor activity in vivo on a milligram per kilogram basis than do the smaller compounds (MW, 12,500–15,500). This MW relationship was observed both when the

TABLE 11.—Combination ^{60}Co-MVE against Lewis lung carcinoma*

Radiation (rads)	MST ± SE with—	
	PBS	MVE-3 (25 mg/kg)
None	17.9 ± 0.9	19.6 ± 2.4
2500	27.0 ± 3.0†	26.0 ± 3.0
3500	29.7 ± 2.6†	27.6 ± 3.7†
4500	32.3 ± 2.6†	31.6 ± 2.3†

*Mice were inoculated with 1×10^6 tumor cells in the footpad and tumors were locally irradiated on Day 7 when tumor volumes averaged 100–200 mm^3. MVE-3 was injected iv on Days 9, 16, 23, 30, 37, and 44.
†$P < 0.05$ in comparison to group who received no radiation.

compounds were tested by themselves for antitumor activity against the P815 mastocytoma and when administered in combination with surgical removal of the primary Lewis lung carcinoma. The present results are similar to those reported for combination chemoimmunotherapy with MVE compounds against the LSTRA tumor (9), and for MVE potentiation of L1210 vaccine-induced resistance (13). While the higher MW compounds (MW, > 20,000) appeared to exhibit more antitumor activity than did the lower MW compounds, all of the polyanions showed significant activity.

With regard to therapy against the Lewis lung tumor, the combination of MVE compounds administered iv twice a week for 3 weeks after surgery produced an effect greater than that of either surgery or MVE treatment alone. An ILS of up to 150% was observed. In contrast to the combination with surgery, the combination of MVE treatment with local radiation to reduce the primary tumor did not produce an additive or synergistic effect over that produced by radiation alone. The combination of conventional chemotherapy with MCCNU and MVE was also ineffective. The ineffectiveness of the combination of MVE compounds with either radiation or MCCNU treatment to reduce the primary tumor is disappointing, particularly with the radiation treatment in which the significant prolongation of survival time produced by radiation was not increased with immunotherapy.

The results from the toxicologic studies reaffirmed the MW- and dose-dependent effects of the MVE polymers and reaffirmed that the lower MW compounds probably differ considerably from the higher MW compounds in pharmacokinetic distribution and thus in therapeutic effects. Although there is a MW relationship between MVE polymers and their acute toxicity, this relationship does not appear to be linear. Death occurs within 3 minutes of iv injection and the nervous system appears to be the target. The mice first show some spinal cord stimulation followed by respiratory suppression. The acute toxicity can be markedly decreased by giving divided doses or a very slow infusion. Ip toxicity values are between three and ten times lower than the iv LD50.

As with many other immunomodulators, ie, *Corynebacterium parvum* (24), *Mycobacterium bovis* strain bacillus Calmette-Guérin (25), and tilorone (26), MVE polymers have the ability to sensitize mice to the lethal action of bacterial endotoxin (21). The target organ for this sensitization and lethal process has not yet been identified. With regard to hematologic effects, MVE-1 and -2 did not cause changes over a 7-day period. The leukocytosis produced by MVE-3 and -4 on Days 3 and 5 and with MVE-5 on Days 1–5 after drug injection was primarily caused by increases in granulocytes and monocytes. This may be due to the mobilization of bone marrow cells in a fashion similar to that produced by bacterial endotoxin (27). Possibly the most significant hematologic change was the thrombocytopenia, which has also been reported in clinical studies (16). Preliminary studies indicate that this returns to normal within 3–5 days after iv injection. It has not yet been determined whether the decrease is caused by platelet consumption or inhibition of platelet production.

Previous reports from our laboratories showed that the polydisperse NSC-46015 produced a biphasic response in RES function (19); high-MW polymers (> 15,000) suppressed RES activity while low-MW polymers (< 5000) enhanced RES activity (15). The patterns of RES response in these studies can be explained by the sole action of the MVE polymers on the Kupffer cells of the liver, which is the major organ for the removal of iv injected SRBC. Inhibition of hepatic phagocytosis prolongs the vascular half-life, providing the macrophages of the lung and spleen with additional exposure to the particle and thus increased uptake. The outcome of this altered distribution of particles in triggering immune responses is not known. However, these varying effects of MVE polyanions of different MW may be related to some of the paradoxical immunomodulatory effects observed with these compounds. Iv injection of the polydisperse NSC-46015 enhanced Friend leukemia virus disease while ip injection inhibited the leukemogenesis (3).

Experiments on the MMFO system demonstrated that MVE produces significant decreases in hepatic MMFO as demonstrated by a marked depression of the metabolism of aminopyrine and aniline by liver microsomes of MVE-treated mice. These results are consistent with our previous findings that larger, polydisperse, MVE polymers inhibited MMFO enzymes as measured by the in vitro metabolism of aminopyrine and aniline, and by the prolongation of hexobarbital-induced sleeping times in mice (1). The results of the present study establish that the lowering of the enzyme activity is accompanied by a comparable decrease in hepatic cytochrome P-450 content. The degree of inhibition increased with increased MW of the polymers, with fractions MVE-4 and -5 producing decreases in drug biotransformation equivalent to or greater than that of NSC-46015. Inhibition of MMFO activity by MVE polymers, in general, also exhibited a dose-dependent relationship over a range of 1–50 mg/kg. MVE-2, -3, -4, and -5 produced an inhibition of MMFO activity and cytochrome P-450 content as early as 24 hours after treatment and these parameters did not re-

turn to control levels until 7–14 days. The status of the MMFO enzymes is important when considering the use of immunomodulators as adjuvants to chemotherapy and in patients who receive other supportive drugs whose pharmacokinetics are directly related to the activity of these enzymes. The present results emphasize the necessity of using carefully characterized polyanions in establishing toxic effects and antitumor activities, and suggest that MVE polymers with MW of 12,500–15,000 may have significant clinical potential.

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Response of Murine Leukemia to Combined BCNU-Maleic Anhydride-Vinyl Ether (MVE) Adjuvant Therapy and Correlation With Macrophage Activation by MVE in the In Vitro Growth Inhibition Assay

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SUMMARY

Maleic anhydride-vinyl ether (MVE) fractions, differing in their molecular weights, were studied in the LSTRA murine leukemia chemotherapy adjuvant system. The high molecular weight MVE parent compound (No. 5) and the low molecular weight derivative (No. 2) demonstrated adjuvant activity and enhanced host resistance to LSTRA leukemia following BCNU chemotherapy. These compounds extended survival from 20%–30% in the BCNU controls to 60%–90% in the BCNU plus MVE-treated groups. The MVE compounds were active when administered simultaneously with BCNU chemotherapy or when administered 6 or 13 days following BCNU chemotherapy. Adjuvant activity was obtained with a single iv or ip injection of MVE given at a dose ranging between 25 and 5 mg/kg. Iv inoculation appeared slightly better than the ip route for inducing adjuvant activity. A microculture growth inhibition assay (GIA) for macrophage activation was also employed utilizing macrophages from animals that had been induced in vivo 6 days after ip injection or by in vitro induction using macrophages from untreated animals co-cultured with MVE. Both the high and low molecular weight MVE compounds produced strong macrophage activation in the GIA as measured by inhibition of DNA synthesis of murine leukemia target cells. Both compounds produced similar levels of cytostasis at macrophage to target cell ratios of 20:1–5:1. In vivo macrophage activation was achieved with 25 mg/kg ip and in vitro induction was obtained with 20 μg/culture. A good correlation was obtained between adjuvant activity and remission in the LSTRA model with both molecular weight fractions of macrophage activation as measured in vitro with the microculture GIA. The GIA appears to offer a rapid, sensitive, and reproducible method for screening potential immunoadjuvants for macrophage activation prior to bioassay experiments.

[Cancer Treat Rep 62:1807–1816, 1978]

Maleic anhydride-vinyl ether 2:1 copolymer (MVE or pyran) is a unique compound which has been shown to markedly enhance host resistance against a series of transplantable murine leukemias including LSTRA (1), MBL-2 (2), and MCAS-10 (3), and against solid tumors including Lewis lung carcinoma (1,4), Madison lung carcinoma M109 (5), B16 melanoma (4), and first-generation transplanted mammary carcinoma (6). MVE has also been shown to potentiate the protective effect of L1210 tumor cell vaccine through immune modulation (7,8).

MVE compounds have been synthesized in different chain lengths and molecular weights (9), with the rationale that smaller molecular weight compounds may maintain immune enhancement activity but not have associated toxicity which has been

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[2]We appreciate the excellent secretarial assistance of Kathy Murphy and Carolyn Fox in the preparation of this manuscript, and we also wish to thank Dr. Michael A. Chirigos of the Division of Cancer Cause and Prevention, National Cancer Institute (Bethesda, Md), for his advice, help, and encouragement during this study and Dr. James L. McCoy of the Division of Cancer Biology and Diagnosis, National Cancer Institute, for reviewing this manuscript.

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observed to a limited degree with the larger molecular weight parent pyran compounds (NSC-46015) (10). The mechanism through which antitumor and immune enhancement activities are mediated by MVE appears to be via macrophage (MØ) activation (11–15).

The purpose of this study was to examine the tumor resistance properties of a series of newly synthesized lower molecular weight fractions of MVE to determine if they were effective adjuvants to chemotherapy against the LSTRA murine leukemia as compared to larger molecular weight compounds as previously reported (1). In addition, the MVE compounds were evaluated for their ability to activate normal MØ following in vivo inoculation of MVE in the peritoneal cavity and subsequent collection of MØ or in vitro assessment of activation in microculture growth inhibition assays (GIA) for MØ activation. Finally, we studied the addition of MVE to normal nonactivated MØ in vitro in an attempt to directly correlate the adjuvant effects of these new MVE with MØ activation in the GIA.

MATERIALS AND METHODS

Mice

Male and female inbred (BALB/c × DBA/2)F_1 ($CD2F_1$) mice weighing 20–24 g (approximately 8 weeks old) were obtained from the production colonies of Sprague Dawley (Madison, Wisc) and used throughout these studies. The animals were housed in plastic cages and given tap water and Purina laboratory chow ad libitum.

Tumors

The Moloney leukemia virus-induced transplantable murine leukemia (LSTRA) of BALB/c mice was obtained from the laboratory of Dr. Michael Chirigos. The LSTRA tumor was passaged at weekly intervals by ip injection of 3×10^6 viable tumor cells suspended in Hanks' balanced salt solution (HBSS) into syngeneic mice. For pyran studies, $CD2F_1$ mice were inoculated sc with 1×10^4 LSTRA tumor cells. A tissue culture adapted form of a Moloney virus-induced murine leukemia (MBL-2) was also obtained from Dr. Chirigos and used as target cells for the microculture GIA described below. These cells were maintained in culture by passage twice weekly (1:200 dilution) in RPMI-1640 supplemented with Hepes buffer (25 mM), gentamicin (50 μg/ml), and 20% fetal calf serum (FCS).

Drug

BCNU was used as a cytoreductive therapeutic agent and was supplied by the Division of Cancer Treatment, National Cancer Institute. BCNU was suspended in a 0.3% solution of hydroxypropyl cellulose (Klucel) and inoculated sc at a dose of 30 mg/kg on Day 7 following the inoculation of LSTRA when the mice were demonstrating systemic LSTRA leukemia by preliminary bioassay experiments. MVE 2:1 copolymers were obtained from Dr. David Breslow of Hercules Research Center (Wilmington, Del) in several molecular weight fractions. Of the MVE compounds supplied, our best technical data were obtained with MVE Nos. 2 and 5. MVE No. 2 has an intrinsic viscosity of 0.215 and an estimated molecular weight of 15,500 daltons, while MVE No. 5 has an intrinsic viscosity of 1.321 and an estimated molecular weight of 52,600 daltons. MVEs were dissolved in phosphate buffered saline and adjusted to pH 7.2 using 1 N NaOH. The compounds were administered by ip and iv routes at doses of 25, 10, and 5 mg/kg on Days 7, 13, or 20 following LSTRA inoculation with or without cytoreductive therapy. All mice in the adjuvant chemotherapy study were palpated and weighed twice weekly for a period of 72–90 days.

Adherent Cell Recovery

Peritoneal exudate cells (PEC) were collected from normal untreated $CD2F_1$ mice or mice inoculated ip 6 days earlier with MVE No. 2 and MVE No. 5 (25 mg/kg) by paracentesis using 15 ml of HBSS supplemented with Hepes (25 mM), gentamicin (50 μg/ml), and preservative-free heparin (10 units/ml). Exudates from ten mice per group were pooled, washed, and resuspended in 10 ml of RPMI-1640 supplemented with 20% FCS. Five-ml aliquots were placed into tissue culture grade Petri dishes (Falcon Plastics, Inc, Oxnard, Calif) and incubated at 37°C in a humidified 5% CO_2 and air atmosphere for 2 hours. Nonadherent cells were decanted and the MØ-rich adherent cells were vigorously washed four times with 5 ml of HBSS. The adherent cells were briefly exposed (2 minutes) to a trypsin-EDTA solution (Grand Island Biological Co, NY) and then gently scraped from the dishes with a soft rubber policeman and cell counts and esterase stains were made. Smears of plastic adherent PEC were stained for nonspecific esterase activity by the method of Yam et al (16) to enumerate MØ. Greater than 96% of the plastic adherent cells stained positive for nonspecific esterase activity (a cytochemical marker characteristic of MØ). MØ suspensions were kept on

ice prior to being added to the GIA to prevent adherence.

$$\% \text{ inhibition} = 1 - \frac{\text{cpm MBL-2 + MVE-activated MØ}}{\text{cpm MBL-2 + normal nonactivated MØ}} \times 100.$$

Microculture GIA for MØ Activation

The microculture GIA for murine MØ activation was modified in principle and methodology from previously published procedures (14,15,17). Briefly, 40,000 MBL-2 target cells from 24-hour cultures were aliquoted in a 0.1-ml volume to quadruplicate wells of a round bottom microtiter plate (No. 76–013–05, Linbro Scientific, Inc, Hamden, Conn) and MØ from untreated or MVE-activated $CD2F_1$ mice were added (0.1 ml) at MØ to target cell ratios of 20:1, 10:1, and 5:1. These cultures were then incubated at 37°C in a humidified 5% CO_2 and air mixture for 48 hours. For in vitro MVE activation studies, MØ from untreated mice and target cells were combined, MVE (20 μg/well) was added (0.05 ml), and the cultures were incubated for 72 hours. All cultures were pulsed for 4–6 hours before termination with 1 microcurie of ^{3}H-TdR (specific activity, 6.7 curies/mmol) and the cells were harvested on glass scintillation paper with a multiple sample harvester (Biomedical Research and Development Laboratories, Inc, Rockville, Md). The amount of incorporated ^{3}H-TdR was determined in a liquid scintillation counter. The percent inhibition of target cell proliferation resultant from MØ activation was calculated by the formula:

RESULTS

The protocol followed for examining the adjuvant effects of the MVE compounds in the LSTRA chemotherapy model was essentially that described by Mohr and associates (1). $CD2F_1$ mice were inoculated sc with 1×10^4 LSTRA leukemia cells and after 7 days when the animals had systemic leukemia (as shown by bioassay experiments using blood and spleen cells), these mice were then treated with BCNU cytoreductive therapy (30 mg/kg). Untreated mice died in 11–15 days with 0 survival while BCNU was curative in 20%–30% of the mice and extended the median survival to 25–49 days. MVE compounds were administered on Day 7, 13, or 20.

The results of adjuvant chemotherapy studies in the LSTRA system using the lower molecular weight MVE compound No. 2 administered by the iv route are shown in figure 1. All mice in the untreated control group died with a median survival time (MST) of 13.5 days. The administration of BCNU chemotherapy on Day 7 resulted in a significant increase in survival (20%) with an MST of 49 days. The combination of BCNU with MVE No. 2 adjuvant therapy on Day 7 resulted in an increase in long-term survivors (30%–80%) which was dose-

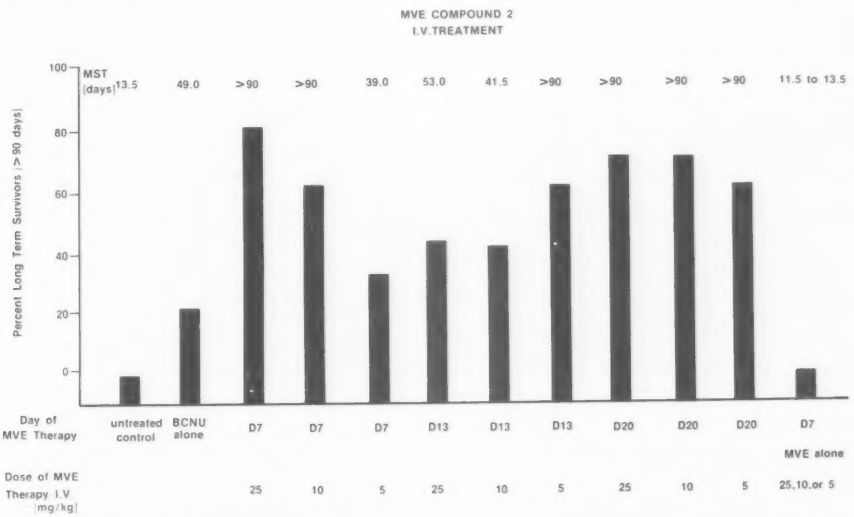

FIGURE 1.—Effect of varying doses and treatment days of MVE No. 2 given by the iv route as an adjuvant to BCNU chemotherapy against LSTRA murine leukemia. LSTRA cells (1×10^4) were inoculated sc into $CD2F_1$ mice (10 mice/group) on Day 0 and BCNU (30 mg/kg) was administered on Day 7 to all groups except untreated controls (20 mice/group) and groups inoculated with MVE alone. Adjuvant therapy with MVE No. 2 was given on Day 7, 13, or 20 at a dose of 25, 10, or 5 mg/kg. The % long-term survivors (> 90 days) is indicated by the vertical bars and MST is indicated above the vertical bars.

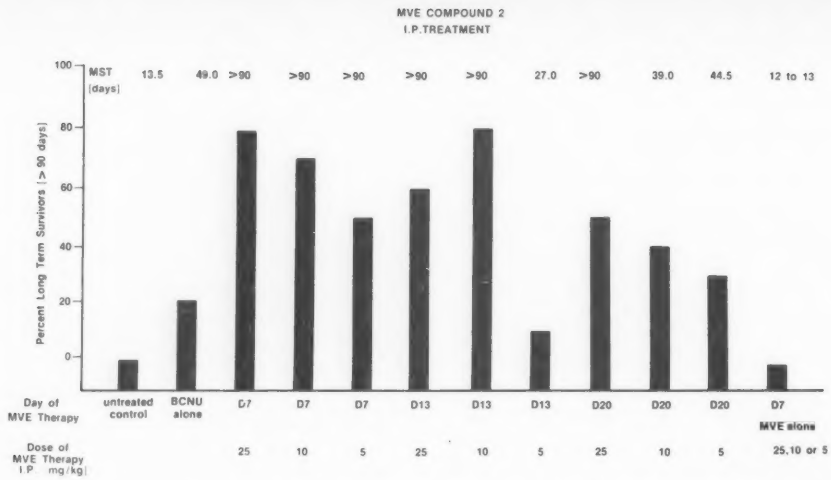

FIGURE 2.—Effect of varying doses and treatment days of MVE No. 2 given by the ip route as an adjuvant to BCNU chemotherapy against LSTRA murine leukemia. LSTRA cells (1×10^4) were inoculated sc into $CD2F_1$ mice (10 mice/group) on Day 0 and BCNU (30 mg/kg) was administered on Day 7 to all groups except untreated controls and groups inoculated with MVE alone. Adjuvant therapy with MVE No. 2 was given on Day 7, 13, or 20 at a dose of 25, 10, or 5 mg/kg. The % long-term survivors (> 90 days) is indicated by the vertical bars and MST is indicated above the vertical bars.

responsive. The MST ranged from > 90 to 39 days at the 5-mg/kg dose level. The application of MVE therapy on Day 13 was less effective and resulted in only 30%–40% survival with an MST range of 39–53 days. The administration of MVE No. 2 as a single dose on Day 20 improved survival, giving 60%–70% long-term survivors at all doses tested. Treatment on Day 7 with MVE alone at all doses did not enhance survival over the untreated controls.

Administration of MVE No. 2 by the ip route (fig 2) gave levels of enhanced survival and reactivity patterns similar to the iv route. Treatment with MVE No. 2 on Day 7 resulted in a dose-response effect with 50%–80% survival and an MST of > 90 days, treatment on Day 13 resulted in 60%–80% survival at all doses except the 5-mg/kg level, and treatment on Day 20 resulted in a diminished response (30%–50% survival). MVE given alone by the ip route at all dose levels was not effective against LSTRA leukemia.

The larger molecular weight MVE No. 5 compound was also tested in the LSTRA system and gave similar results (fig 3). Untreated mice died with an MST of 14 days while BCNU treatment resulted in 30% survivors with an MST of 25 days. Mice given BCNU on Day 7 in combination with MVE No. 5 by the iv route resulted in enhanced survival ranging from 80% to 100%. Day 13 treatment with MVE No. 5 gave a dose-response pattern resulting in 60%–80% long-term survivors. Treatment on Day 20 with MVE resulted in survivals ranging from 20% to 60%. Again, MVE alone on Day 7 was ineffective at all doses with the MST ranging from 11.5 to 13 days, similar to the untreated controls.

When MVE No. 5 was administered by the ip route, it appeared less efficacious than by the iv route (fig 4). MVE given on Day 7 in combination with BCNU was moderately effective with long-term survivors (> 72 days) ranging from 60% to 90% at all dose levels (25, 10, and 5 mg/kg). Treatment on Day 13 also gave long-term survivors ranging from 60% to 90% with a flat dose-response pattern. Treatment with MVE No. 5 on Day 20 resulted in 90% survival at the 25-mg/kg dose level as compared with only 40% survival at the two lower dose levels. In addition, treatment of mice with MVE alone did not enhance survival (MST = 14–15 days). Thus, the in vivo data in the LSTRA system indicate that both MVE fractions which differed by greater than threefold molecular weight had strong immunoadjuvant activities following cytoreductive therapy with BCNU: the new low molecular weight compound appeared as effective as the larger molecular weight parent compound.

The next phase of study was directed at examining the mechanism of action of the MVE compounds and determining if they were acting through a mechanism of MØ activation. These studies were accomplished by the in vitro ip inoculation of normal $CD2F_1$ mice with the MVE compounds followed by a

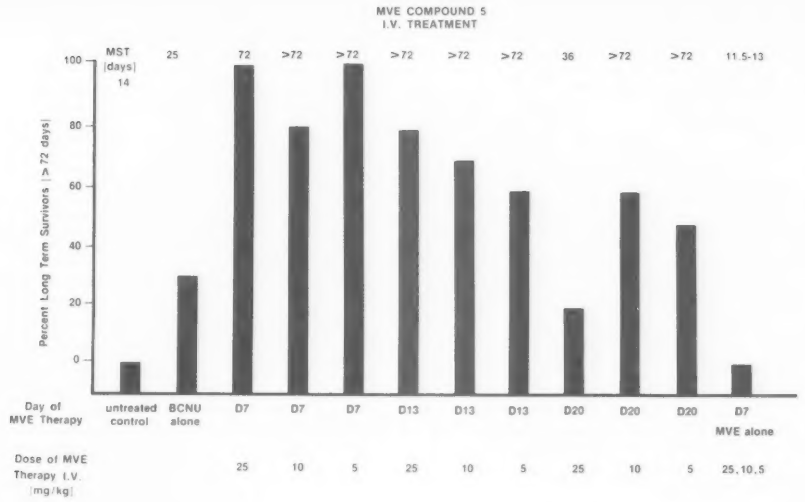

FIGURE 3.—Effects of varying doses and treatment days of MVE No. 5 given by the iv route as an adjuvant to BCNU chemotherapy against LSTRA murine leukemia. LSTRA cells (1×10^4) were inoculated sc into $CD2F_1$ mice (10 mice/group) on Day 0 and BCNU (30 mg/kg) was administered on Day 7 to all groups except untreated controls (20 mice/group) and groups inoculated with MVE alone. Adjuvant therapy with MVE No. 5 was given on Day 7, 13, or 20 at a dose of 25, 10, or 5 mg/kg. The % long-term survivors (> 72 days) is indicated by the vertical bars and MST is indicated above the vertical bars.

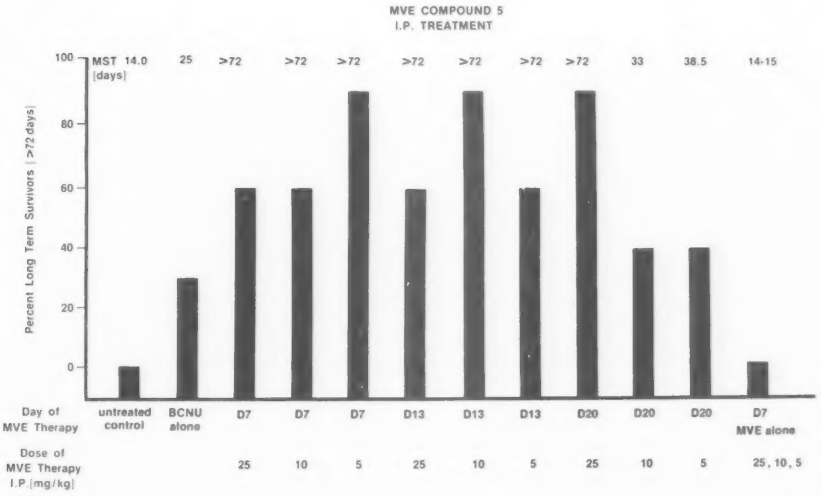

FIGURE 4.—Effects of varying doses and treatment days of MVE No. 5 given by the ip route as an adjuvant to BCNU chemotherapy against LSTRA murine leukemia. LSTRA cells (1×10^4) were inoculated sc into $CD2F_1$ mice (10 mice/group) on Day 0 and BCNU (30 mg/kg) was administered on Day 7 to all groups except untreated controls and groups inoculated with MVE alone. Adjuvant therapy with MVE No. 5 was given on Day 7, 13, or 20 at a dose of 25, 10, or 5 mg/kg. The % long-term survivors (> 72 days) is indicated by the vertical bars and MST is indicated above the vertical bars.

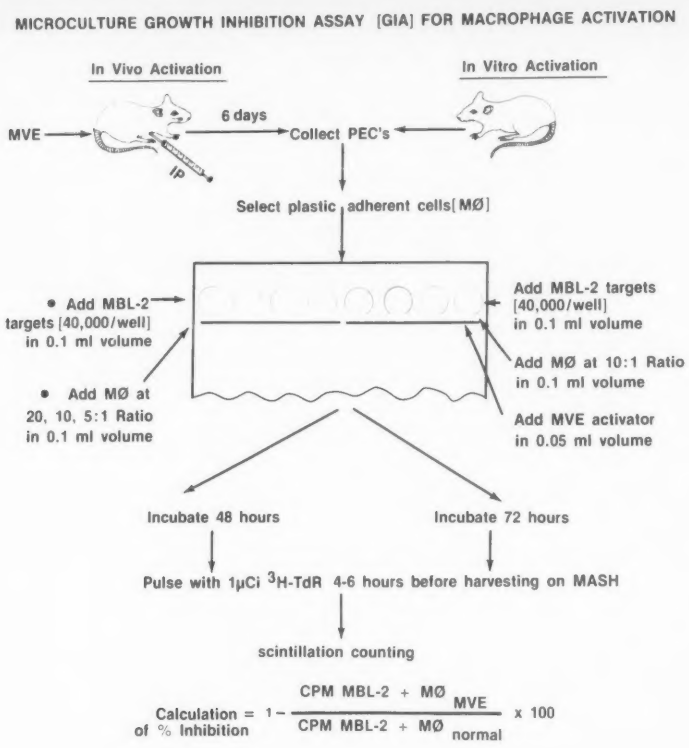
MICROCULTURE GROWTH INHIBITION ASSAY [GIA] FOR MACROPHAGE ACTIVATION
In Vivo Activation
In Vitro Activation
MVE
6 days
Collect PEC's
IP
Select plastic adherent cells[MØ]
• Add MBL-2 targets [40,000/well] in 0.1 ml volume
• Add MØ at 20, 10, 5:1 Ratio in 0.1 ml volume
Add MBL-2 targets [40,000/well] in 0.1 ml volume
Add MØ at 10:1 Ratio in 0.1 ml volume
Add MVE activator in 0.05 ml volume
Incubate 48 hours
Incubate 72 hours
Pulse with 1μCi 3H-TdR 4-6 hours before harvesting on MASH
scintillation counting
Calculation of % Inhibition = 1 − CPM MBL-2 + MØ MVE / CPM MBL-2 + MØ normal x 100

FIGURE 5.—Schematic for microculture GIA for MØ activation following in vivo and in vitro activation of MØ by MVE compounds.

6-day incubation in vivo (in vivo induction) after which the MØ were assayed for activation in a microculture GIA. MØ from unstimulated mice were mixed directly with MVE in vitro (in vitro induction) in the GIA to determine if the MØ could be directly activated in vitro by MVE.

The methodology and procedures used in these studies are diagramatically represented in figure 5. The left portion of this figure deals with in vivo induction of MØ activation while the right portion deals with the in vitro induction of MØ activation by the direct mixing of MVE with unstimulating MØ.

Data from the in vivo induction of MØ activation by MVE compounds as measured by the GIA are presented in table 1. MVE No. 2 at a 20:1 ratio of MØ to MBL-2 target cells induced the highest levels (49%) of growth inhibition of MBL-2, although significant levels of inhibition were also observed at the 10:1 and 5:1 ratios. In contrast, MVE compound No. 5 produced the highest level of growth inhibition (64%) at the 10:1 ratio. In addition, significant levels of growth inhibition were also obtained at the 20:1 and 5:1 MØ to MBL-2 target cell ratios. It should be noted that all ratios of MVE-activated MØ to MBL-2 target cells were compared against similar ratios of MØ from untreated mice co-cultured with MBL-2 for calculating percent inhibition or levels of significance (data not shown). Thus, these data indicate that both MVE compounds induced similar levels of MØ activation following a 6-day in vivo exposure of peritoneal MØ. These data further support the concept that the mechanism for the adjuvant activity of MVE is through MØ activation in vivo following the administration of MVE by either the iv or ip route.

We next focused on the in vitro induction of MØ activation by MVE compounds to determine if in vivo exposure of MØ to MVE is required for activation. The data from the in vitro induction experiments are shown in table 2. The MVE compounds were co-cultured for in vitro activation studies at several concentrations in the presence of MØ and were also assayed in the absence of MØ, directly on MBL-2 target. In these studies, 20 μg/well (equivalent to 100 μg/well in a 1-ml culture) gave the

TABLE 1.—In vivo induction of MØ activation by MVE compounds as measured by in vitro microculture GIA

Treatment in vivo*	MØ:MBL-2 ratio†	cpm ^{3}H-TdR‡ (± SE)	% inhibition§	Level of significance¶
MVE 2	20:1	5570 (367)	49 ↓	< 0.001
MVE 2	10:1	8653 (346)	21 ↓	< 0.01
MVE 2	5:1	7610 (447)	30 ↓	< 0.01
MVE 5	20:1	6800 (250)	38 ↓	< 0.001
MVE 5	10:1	3880 (126)	64 ↓	< 0.001
MVE 5	5:1	5807 (196)	50 ↓	< 0.001

*All mice were injected ip with 25 mg/kg and PEC were collected after 6 days from 10 mice/treatment group.

†Ratio of plastic adherent cells (> 96% MØ) to MBL-2 target cells plated at 40,000 cells/well. All treatment ratios were performed in quadruplicate microcultures.

‡Mean cpm of ^{3}H-TdR incorporation (± SE) following a 6-hr pulse and terminated after 48 hrs of incubation.

§% inhibition of ^{3}H-TdR incorporation in MBL-2 target cells was calculated by the following formula:

$$\% \text{ inhibition} = 1 - \frac{\text{cpm MBL-2 + MØ from MVE-treated mice}}{\text{cpm MBL-2 + MØ from untreated mice}} \times 100.$$

¶Level of significance computed by Student's t test by comparing mean of cpm from MØ of untreated mice at each MØ:MBL-2 ratio to MVE-activated MØ.

maximum level of activation at the 10:1 ratio of MØ to MBL-2 targets. Using MVE No. 2 at the 20-μg/well dose we observed 50% inhibition of DNA synthesis in MBL-2 leukemia cells at the 10:1 ratio which was highly significant ($P < 0.001$). The addition of MVE No. 2 directly to MBL-2 in the absence of MØ was not inhibitory, but slightly enhanced (12%) ^{3}H-TdR incorporation. MVE No. 5 at the 20-μg/well dose at the 10:1 ratio gave similar, but slightly less, levels of MØ activation resulting in 41% inhibition ($P < 0.001$). MVE No. 5 had no direct effect on MBL-2 targets in the absence of MØ. Thus, these data indicate that culturing of MØ in vitro with MVE was adequate to induce activation. Good agreement was found between in vivo or in vitro induction of MØ activation by MVE compounds and adjuvancy effects, thus indicating that there is no apparent requirement for in vivo induction.

DISCUSSION

The data presented here confirm and extend the observation of Mohr et al (1), who observed that MVE (NSC-46015) actively induced host resistance and increased the percentage of long-term survivors of LSTRA-bearing mice following BCNU chemotherapy. Compounds with molecular weights higher and lower than the parent compound were also found to show some antitumor activity in their studies, which indicated that MVE was active over a broad molecular weight range. MVE has also been shown to have adjuvant activity in other murine leukemia systems (2,3). Studies with MVE have indicated that toxicity is a function of increasing molecular weights (9). Thus, the purpose of the present study was to examine compounds of lower molecular weight than the parent molecule to determine if toxicity could be circumvented while retaining the tumor resistance activity of the parent molecule in the LSTRA adjuvant chemotherapy system.

The data indicate that MVE compound No. 5 which resembles the parent compound in molecular weight and MVE No. 2 which is approximately one fourth the molecular weight of MVE No. 5 were both highly active adjuvants in increasing host resistance and the percentage of long-term survivors following BCNU chemotherapy in animals bearing systemic LSTRA leukemia. Similarly, both compounds gave comparable levels of increased survival and remission. The two compounds appeared more efficacious by iv rather than ip inoculation.

We also observed that both MVE compounds were

TABLE 2.—In vitro induction of MØ activation by MVE compounds as measured by in vitro microculture GIA

Treatment in vitro*	Concentration†	MØ:MBL-2 ratio‡	cpm ^{3}H-TdR§ (± SE)	% inhibition¶	Level of significance**
None*	0	10:1	10,927 (523)	—	—
MVE 2	20 μg/well	10:1	5472 (640)	50 ↓	< 0.001
MVE 2	20 μg/well	—	12,229 (319)	12 ↑	NS
MVE 5	20 μg/well	10:1	6481 (513)	41 ↓	< 0.001
MVE 5	20 μg/well	—	10,688 (634)	2 ↓	NS

*PEC were collected from normal untreated $CD2F_1$ mice and plastic adherent cells were prepared (> 96% esterase positive).

†MVE diluted in complete media was added (0.05 ml) to treatment wells containing MØ and to control wells containing only MBL-2 target cells.

‡Ratio of plastic adherent MØ to MBL-2 target cells plated at 40,000 cells/well. All treatments were performed in quadruplicate microcultures.

§Mean cpm of ^{3}H-TdR incorporation (± SE) following a 6-hr pulse and terminated after 72 hrs of incubation.

¶ $\%\ \text{inhibition} = 1 - \frac{\text{cpm MBL-2 + MØ with MVE}}{\text{cpm MBL-2 + MØ without MVE}} \times 100.$

**Level of significance computed by Student's t test by comparing mean of cpm from MVE-treated MØ to cpm from untreated MØ.

potent adjuvants even when used simultaneously with BCNU chemotherapy on Day 7. This observation is slightly discordant with earlier data (1) where optimum results were obtained on Day 13 following either single or multiple injections, although in that study extensive experiments were not performed using adjuvant therapy concomitant with chemotherapy. One possible explanation for the ability of MVE to demonstrate activity during the chemotherapy phase is that MVE compounds are probably selecting a much different target cell for activity than is the cytoreductive agent BCNU, which is directed primarily against leukemia target cells and normal lymphoid components of the host. It should also be noted that in this study, MVE Nos. 2 and 5 were less active on Day 13 than on Day 7. This may be related to an active proliferation and repopulation of lymphoid cells on Day 13 following BCNU chemotherapy. It is also obvious from our data that a single inoculation of MVE adjuvant therapy is adequate to affect a cure and produce remission in a high proportion of LSTRA-bearing animals.

Survival data reminiscent of a dose response with MVE were observed at certain inoculation times. These data are consistent with earlier observations reporting that dose-response curves were difficult to obtain with MVE compounds in LSTRA (1). Toxic effects in the form of death or an observable abnormal behavior were not associated with either of the MVE compounds.

MVE compounds were not active in our study when used alone in the absence of cytoreductive therapy with BCNU. Thus, it would appear that one needs to first reduce the tumor burden in order to demonstrate the adjuvancy effects of the MVE compounds, an observation which is consistent with the earlier studies of Mohr et al (1) who demonstrated that the MVE compounds were not active against LSTRA without first administering cytoreductive chemotherapy.

The mechanism of action by which MVE compounds enhance host resistance against tumor appears to be mediated through the activation of MØ in a nonspecific manner resulting in the inhibition of DNA synthesis or cytolysis of neoplastic cells (2,5,12,14,15). Further studies by Kaplan et al (12) indicate that MØ activation by MVE is independent of T-cell immunocompetency, since irradiated and bone marrow reconstituted mice, with no evidence of T-cell reactivity, produced cytotoxic MØ when challenged with MVE. Further evidence for the T-cell independency of the MØ activation process comes from data of Schultz et al (14) in which athymic nude mice (which have no evidence of T-cell function) were injected ip with MVE, and the

MØ harvested 6 days later and assayed in vitro in a cytotoxicity assay against MBL-2 cells were strongly cytotoxic against the leukemia target cells.

MØ are further implicated in enhanced host resistance following administration of MVE from our in vitro studies in which MVE added to MØ and leukemia target cells resulted in cytostasis and inhibition of tumor cell proliferation (15). Direct contact was also required between MØ and target cells since cytotoxic factors could not be identified in supernatants from activated MØ cultures (14). Studies also indicate that MVE compounds are rapidly assimilated by the MØ as shown by ^{14}C labeling studies (18). In vivo and in vitro induction studies indicate that over a period of 24–72 hours following MVE exposure cytostatic and cytotoxic MØ are induced. Thus, published evidence corroborates the findings of our studies and indicates that MVE compounds are active through the mechanism of MØ activation.

Data from our laboratory using a modified microculture GIA for MØ activation indicate that both MVE Nos. 2 and 5 actively induced MØ activation when administered ip to $CD2F_1$ mice and when the MØ were harvested 6 days later. Both molecular weight fractions exhibited similar levels of inhibition of DNA synthesis (cytostasis) over several MØ to MBL-2 target ratios using an in vivo dose of 25 mg/kg. It is also apparent from the in vivo MVE induction study that both compounds induced strong MØ activation as demonstrated in the GIA. Neither of the MVE fractions alone was directly inhibitory to the growth of MBL-2 in the GIA. These data are consistent with the findings of Schultz et al (2) who observed MØ activation following the in vivo ip administration of the parent MVE compound (NSC-46015) and observed that this reactivity peaked on Day 6 in the peritoneal MØ population.

The final phase of our study focused on determining if the newly synthesized MVE fractions could directly activate normal MØ in vitro. We observed MVE Nos. 2 and 5 activated MØ to inhibit DNA synthesis and proliferation in MBL-2 target cells. MØ alone or MVE alone failed to induce suppression of DNA synthesis which indicated that the MVE compound was acting via the activation of MØ. Both molecular weight fractions gave similar levels of GIA activity suggesting that the lower molecular fraction was as potent an MØ activator as the larger molecular fraction.

Our data suggest that enhanced host resistance to LSTRA observed with MVE compounds in the BCNU adjuvant chemotherapy system was probably mediated by mechanisms of MØ activation. Both in vivo and in vitro induction studies with the two MVE fractions were shown to induce potent MØ mediated tumor cell cytostasis. Finally, we believe that the microculture GIA represents a rapid, sensitive, and reproducible screening procedure for defining compounds with potential MØ activation properties which should be used before implementing more costly and cumbersome bioassay studies in animals.

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Effect of Maleic Anhydride-Divinyl Ether Copolymers on Experimental M109 Metastases and Macrophage Tumoricidal Function

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SUMMARY

Pyran copolymer (NSC-46015) was compared with five maleic anhydride-divinyl ether copolymers (MVEs) of narrow molecular weight range both for the ability to render macrophages nonspecifically tumoricidal and to retard the development of artificially induced metastases. All MVEs were found effective at activating macrophages in vivo, although the optimal dose for each varied. No correlation was obtained between intrinsic viscosity and degree of activation. Pyran was found to strikingly inhibit M109 pulmonary metastases formation when given over a period of 5 days prior to, or 1 day after, iv tumor inoculation. Histologically, tumor inhibition appeared to result from macrophage accumulations and histiocytic granulomas in the lung. Generally, when MVEs were compared in the artificial metastasis model, polymers with the lower molecular weights were the most active.

[Cancer Treat Rep 62:1817–1822, 1978]

During the past few years, our laboratory has established the beneficial effects of pyran copolymer against several experimental tumors. Pyran has been demonstrated to be a potent activator of macrophages, possibly acting through an interferon induction mechanism (1). This activation does not appear to be dependent on T-lymphocyte collaboration, since cytotoxic macrophages have been recovered from athymic nude mice treated with pyran (2).

Previous experiences showed that the early clinical pyran (NSC-46015), as a single treatment modality, enhanced macrophage tumoricidal activity against the Madison 109 lung carcinoma (M109) and enhanced host survival (3,4). In addition, ip pyran treatment has also been shown to potently inhibit the number of lung metastases that develop after iv inoculation of single-cell suspensions derived from the M109 tumor (5). In this paper, we compare the activity of various intrinsic viscosities of maleic anhydride-divinyl ether copolymers (MVEs) on growth retardation of artificially induced lung metastases in syngeneic BALB/c mice and attempt to correlate this activity with the presence of nonspecifically cytotoxic macrophages.

MATERIALS AND METHODS

Mice.—Male BALB/c mice, 6–8 weeks old, were obtained from the Mammalian Genetics and Animal Production Section, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Mice were housed in plastic cages with air filter bonnets and fed Purina laboratory chow and tap water ad libitum.

Drugs.—Pyran copolymer (NSC-46015) and MVE copolymers of narrow molecular range were kindly supplied by Dr. David Breslow of Hercules Research Center (Wilmington, Del). Drugs were dissolved in Dulbecco's phosphate-buffered saline, adjusted to pH 7.0 by the addition of 0.1 N NaOH, and given ip at 1% of the body weight.

Cell culture.—An established cell line of M109 ($H2^d$, BALB/c-derived) murine alveolar carcinoma cells was maintained in RPMI-1640 medium supplemented with 20% heat-inactivated (56°C for 30 min-

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[3] We wish to thank Douglas C. Jones, Division of Cancer Cause and Prevention, National Cancer Institute, for photographic assistance.

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utes) fetal calf serum (FCS), 100 μg/ml of gentamicin solution, 0.075% $NaHCO_3$, and 10 mM Hepes buffer (RPMI-FCS). These cells were used both for target cells and as a source of tumor cells for the experimental metastasis assays. The properties of the M109 cell line have previously been described (6).

Peritoneal macrophages.—Noninduced peritoneal macrophages were harvested and purified by adherence as previously described (2). Representative preparations of purified adherent cells were stained with Giemsa stain; > 95% of the cells observed had morphologic characteristics of macrophages. Macrophages were kept in an ice bath prior to use to prevent adherence.

Test for activation of macrophages in vivo.—The ability of activated macrophages to diminish target cell proliferation was measured by the inhibition of DNA synthesis (IDS) assay previously described (3). Briefly, target cells were trypsinized from exponentially growing cultures and resuspended at 5.0×10^4 cells/ml of RPMI-FCS, and 2-ml aliquots were placed in 30-mm tissue culture dishes. Purified peritoneal macrophages were adjusted to 1.0×10^6 cells/ml of RPMI-FCS, and 1 ml was added to the target cell cultures. DNA synthesis of the target cells was assessed after 20 hours of incubation at 37°C. Triplicate cultures of each set of dishes, as well as cultures consisting of tumor cells alone and macrophages alone, were pulsed with 2.0 microcuries of tritiated thymidine (specific activity, 10 curies/mmol) for 2 hours at 37°C. At the end of the incubation, the cells were detached with trypsin, centrifuged at 600 × *g* for 10 minutes, and resuspended in 0.5 ml of Dulbecco's phosphate-buffered saline. Four milliliters of chilled 10% trichloroacetic acid (TCA) was added to each tube, and precipitation was allowed to continue for 30 minutes at 4°C. The resultant precipitate was collected on glass-fiber paper and washed with cold 10% TCA. The filters were air-dried and assayed for radioactivity using Aquasol solubilizer (New England Nuclear Corp, Boston, Mass). The percent IDS was calculated by the following formula:

$$\% \text{ IDS} = \frac{\text{cpm}_N - \text{cpm}_E}{\text{cpm}_N} \times 100$$

where cpm_N = mean counts per minute in cultures containing effector cells from normal control mice and cpm_E = mean counts per minute in cultures containing test effector cells.

Inhibition of artificially induced lung metastases.—M109 cells grown in vitro were harvested during their exponential growth phase by gentle trypsinization, washed twice, and resuspended in serum-free RPMI-1640 medium. The number of single viable cells was determined and adjusted to 1×10^5 cells/ml of medium. Tumor cells were injected iv in normal BALB/c mice. The inoculum volume per mouse was 0.2 ml (2×10^4 cells). Drugs were given ip to randomized groups of mice at various time intervals with respect to tumor cell inoculation. Controls received 0.2 ml of Dulbecco's phosphate-buffered saline. Five mice from each group were killed on Days 14 and 20, and the number of pulmonary metastases was determined by India ink inflation by the technique of Wexler (7).

Histopathology.—Lungs were excised at Day 14 after iv M109 tumor inoculation and fixed in 10% neutral formalin. These tissues were sectioned and routinely stained with hematoxylin and eosin.

RESULTS

Effect of pyran and MVE copolymers on macrophage activation in vivo.—The MVEs of differing intrinsic viscosities were compared with pyran for the ability to activate murine peritoneal macrophages in vivo. The drugs were administered ip at either 10, 25, or 50 mg/kg. Macrophages were harvested 6 days later and tested for their ability to inhibit M109 DNA synthesis. No correlation was observed between intrinsic viscosity of the MVE copolymers and functional activity (fig 1). All MVEs were effective at producing cytostatic macrophages although the optimal concentration for each drug varied.

Effect of pyran on artificially induced pulmonary metastases.—Mice were treated ip with pyran at 25 mg/kg at various times with respect to iv tumor inoculation. The mice were killed on Days 14 and 20, and the number of lung metastases was determined by India ink inflation. The results in table 1 show that pyran almost completely suppressed the development of metastatic lung lesions when given as a single ip injection 5 days prior to tumor inoculation. In contrast to multiple tumors in the lungs of placebo-treated animals, many lungs from pyran-treated mice remained tumor-free on Day 20 (fig 2). Potent antitumor activity was also demonstrated when pyran was given over a period of 3 days prior to, or 1 day after, tumor inoculation (table 1).

Host response to tumor.—A comparative histopathologic study was performed on the lungs from BALB/c mice that had previously received either pyran or 0.9% NaCl solution treatment 5 days prior to iv tumor inoculation. By Day 14, small tumor cell complexes were found within the lung parenchyma from placebo-treated mice. There was no obvious reaction against the tumors from the surrounding host tissue (fig 3). The number of interstitial cells was normal in the alveolar wall (septa) (fig 4). In contrast, grossly and histologically, no tumor cells could be detected in pyran-treated lungs, and there

TABLE 1.—Inhibition of artificially induced lung metastases of M109 lung carcinoma by pyran copolymer (NSC-46015)*

Experiment No.	Drug treatment	Mean No. of lesions ± SE/lung (Day 14)	No. of mice with lung tumors/ total No. of mice	Mean No. of lesions ± SE/lung (Day 20)	No. of mice with lung tumors/ total No. of mice
1	Control	30.8 ± 11.7	5/5	> 250	5/5
	Pyran† (Day –5)	0.2 ± 0.2	1/5	1.2 ± 0.7	3/5
	Pyran (Day –3)	0.2 ± 0.2	1/5	10.4 ± 2.9	5/5
	Pyran (Day 0)	2.4 ± 0.5	5/5	11.6 ± 3.0	5/5
	Pyran (Day 1)	3.8 ± 1.0	5/5	63.5 ± 20.4	5/5
2	Control	4.0 ± 0.4	5/5	117.0 ± 54.3	5/5
	Pyran (Day –5)	0.6 ± 0.4	2/5	0.4 ± 0.2	2/5
	Pyran (Day 0)	0.0 ± 0.0	0/5	1.6 ± 1.1	3/5
	Pyran (Day 1)	2.6 ± 0.6	5/5	2.2 ± 1.3	5/5
3	Control	9.4 ± 1.8	5/5	> 250	5/5
	Pyran (Day –5)	1.2 ± 0.7	1/5	7.4 ± 4.6	2/5

*2×10^4 M109 cells were injected iv on Day 0. Lung lesions were identified by inflation of India ink.
†Pyran was administered ip at 25 mg/kg on day indicated.

was an extensive infiltration of the alveolar septa by histiocytic cells (fig 5). The alveolar septa appeared broadened because of these increased histiocytic cells, and focal accumulations of these cells led to the appearance of "histiocytic granulomas" (fig 6).

Effect of MVEs on experimental metastasis model.—The MVE copolymers of differing intrinsic viscosities were tested against artificially induced M109 metastases. Drugs were administered ip 5 days prior to tumor inoculation. The MVE copolymers with the lowest molecular weights (MVE-1, MVE-2, and MVE-3) were active at inhibiting the number of developing metastatic lung lesions by Day 20 (fig 7). MVE-2 demonstrated the most potent suppression of tumor nodule development.

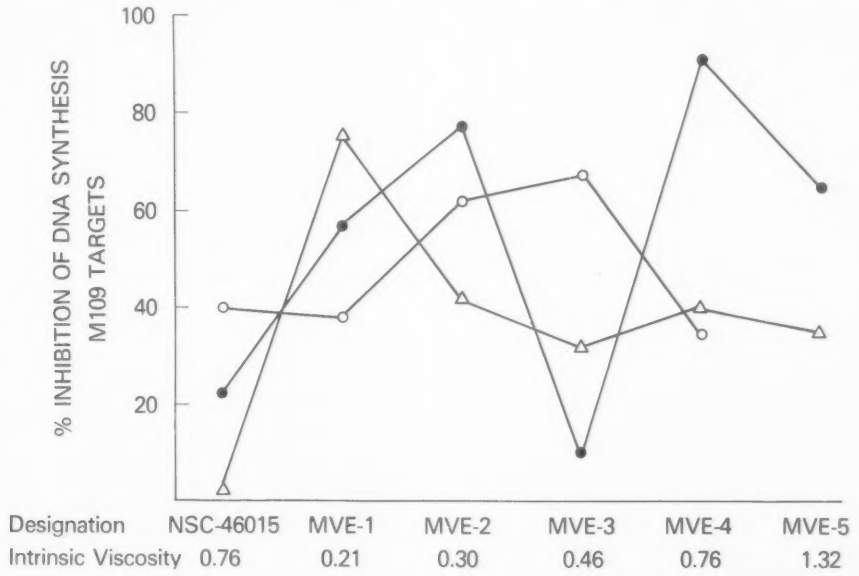

FIGURE 1.—Dose dependency of macrophage activation in vivo by pyran and MVE copolymers. Macrophages were harvested 6 days after ip drug administration at either 10 (●), 25 (○), or 50 (△) mg/kg and tested for their ability to inhibit M109 cell proliferation. Values represent the means of triplicate samples.

FIGURE 2.—Effect of pyran copolymer treatment on artificially induced M109 pulmonary metastases. Mice were treated ip with pyran at 25 mg/kg 5 days prior to receiving 2×10^4 M109 cells iv. The upper figure shows India ink-inflated lungs from placebo-treated animals on Day 20. Numerous tumor lesions are visualized by India ink treatment. In contrast, the lower figure shows lungs that are essentially free of identifiable tumor nodules from pyran-treated animals.

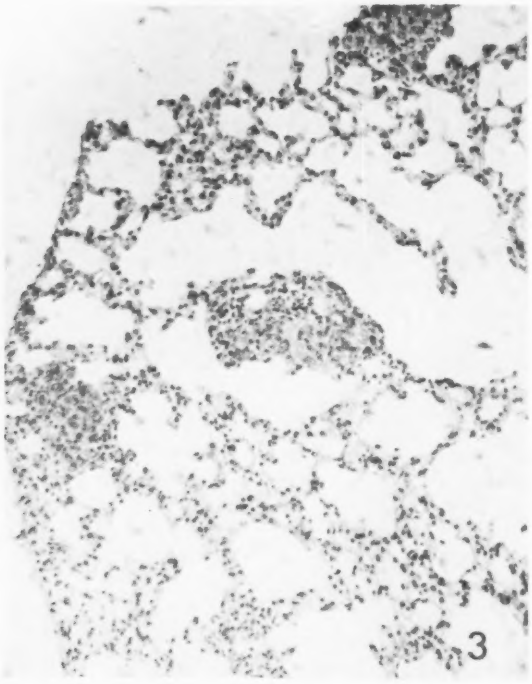

FIGURE 3.—Tumor cell complexes within the lung parenchyma, revealing no reaction from host, 19 days after placebo treatment (× 125).

FIGURE 4.—Alveolar wall (septa) with normal number of interstitial cells 19 days after placebo treatment (× 250).

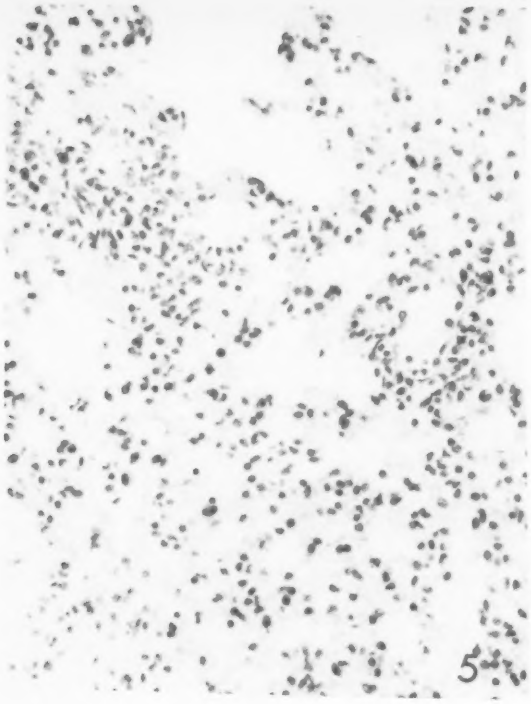

FIGURE 5.—Broadening of alveolar septa with an increased number of histiocytic cells 19 days after pyran treatment (× 250).

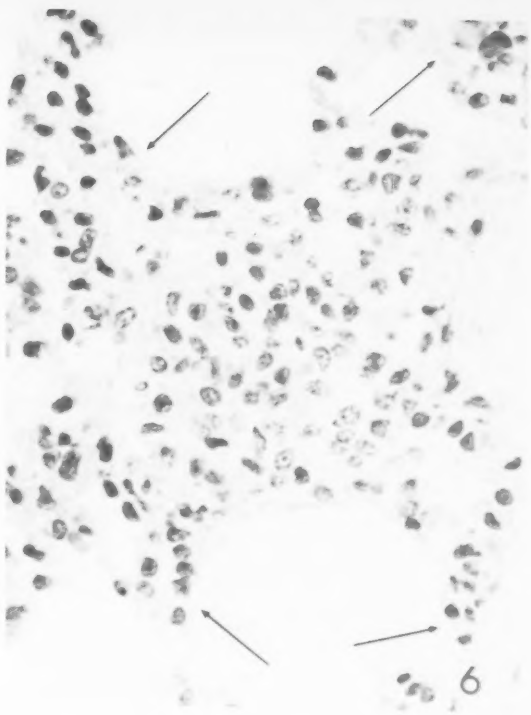

FIGURE 6.—Histiocytic granuloma (aggregate of tightly packed histiocytes) within alveolar wall. Increased number of histiocytes in radiating alveolar septa (arrows) 19 days after pyran treatment (× 500).

DISCUSSION

A large number of biologic and synthetic agents exist which are capable of modulating host immunity against neoplasia. Because activated macrophages can selectively kill transformed cells (8) and the ability of immunoadjuvants to enhance host resistance to cancer correlates with their ability to render macrophages cytotoxic (4), nonspecific macrophage activation appears to be a very promising approach in the management of neoplastic disease. Pyran copolymer has been demonstrated to be one of the most active macrophage stimulants in our hands (4,9). In addition, it has been shown to increase the number of histiocytes (macrophages) infiltrating the sc M109 lung carcinoma (3) and No. 26 colon carcinoma (4) when inoculated at a distant site. In this regard, it increases both the macrophage functional activity and the effector ratio at the distant tumor site.

In this report, we show that pyran strikingly inhibited M109 pulmonary metastases formation when given over a period of 5 days prior to, or 1 day after, iv tumor inoculation. Histologically, tumor inhibition appeared to result from macrophage accumulations in the lung similar to what has been observed for sc tumors (3,4). The mechanism by which pyran increases the mobilization and deposit of macrophages at the tumor site is not known. In addition, we compared the MVEs of narrow molecular weight range for their ability to activate macrophages and inhibit M109 experimental metastases. It was hoped that an MVE with smaller molecular weight, and thus less inherent toxicity (10), would retain good antitumor activity. All MVEs were found effective at activating macrophages in vivo, although the optimal concentration for each varied. In the artificial metastasis model, the polymers with the lower molecular weight were observed to be the most active, with MVE-2 demonstrating the strongest tumor inhibition. However, more testing is required at different dose levels to determine which MVE is most beneficial.

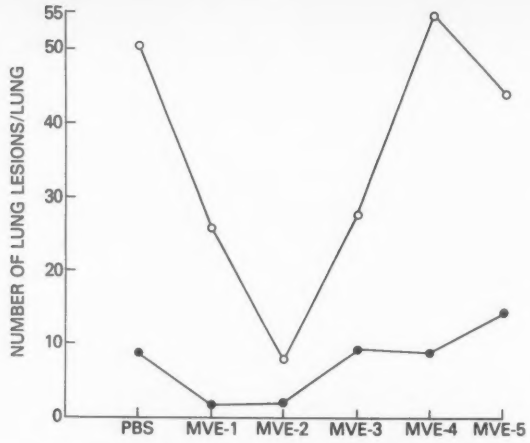

FIGURE 7.—Comparison of MVE copolymers for ability to inhibit experimental M109 metastases. MVEs were inoculated ip at 25 mg/kg 5 days prior to receiving tumor cells. 2×10^4 M109 cells were inoculated iv, and the number of lung lesions was determined by India ink inflation on Day 14 (●) and Day 20 (○). Values represent the mean number of lung lesions from 5 lungs.

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Potential Mechanism of Macrophage-Mediated Tumor Cell Cytotoxicity [1]

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SUMMARY

Activated macrophages have been shown to inhibit the incorporation of ^{125}IUDR into the DNA of Lewis lung (LL) tumor cells and to lead to the cytotoxicity of these target cells. Despite this apparent inhibition of DNA synthesis, the increase in the number of LL cells cultured with activated macrophages paralleled that of LL cells alone for the first 18 hours of culture (approximately one doubling of cells). To resolve this paradox, the DNA content of LL cells, which were cultured with activated macrophages for 16 hours, was analyzed by flow microfluorometry. These experiments indicated that subsequent to co-culture with activated macrophages, LL cells with a 50% reduction in their normal DNA content could be detected. Furthermore, after a 2-hour incubation with colcemid, a > 95% reduction in metaphase cells occurred in LL cells cultured with activated macrophages. However, dividing nuclei without chromosome condensation appeared to be a prominent feature of these cultures. This would suggest that the genotypic or phenotypic program for cell division in the tumor cells is such that under these unique circumstances of tumor cell-activated macrophage interaction, cell division can proceed in the absence of DNA synthesis. We suggest that this aberrant division may be related to the lethal event which leads to activated macrophage-mediated tumor cell cytotoxicity.

[Cancer Treat Rep 62:1823–1829, 1978

Macrophages[4] activated in vivo with reticuloendothelial stimulants such as pyran copolymer, *Mycobacterium bovis* strain bacillus Calmette-Guérin, or *Corynebacterium parvum*, or in vitro with lymphokines have been shown to exhibit cytostatic and/or cytotoxic effects against target tumor cells (1–7).

The preponderance of data indicates that cytotoxicity mediated by activated macrophages is generally selective for neoplastic cells and requires cell-to-cell contact (2–4). Hibbs (2) and Bucana et al (8) have suggested that cytotoxicity is mediated by the transfer of lysosomes from activated macrophages to tumor cells and their subsequent rupture within the tumor cell leading to heterocytolysis. In contrast, Currie and Basham (9) have suggested that cytotoxicity may be mediated by a labile factor inhibited by fetal calf serum. Other data have indicated that activated macrophages inhibit incorporation of ^{125}IUDR or ^{3}H-thymidine into tumor cells and block normal metaphase formation (10–12). In contrast, cinemicrophagic analysis of activated macrophage tumor cell cultures indicated that the target tumor cells divided before cytotoxicity occurred (13). This paradox led us to consider the hypothesis that, prior to the cytotoxic event, tumor cells in the presence of activated macrophages

[1]Supported in part by Public Health Service grants CA-16059 (National Cancer Institute) and AI-70863 (National Institute of Allergy and Infectious Diseases, Dr. Morahan) from the National Institutes of Health, Department of Health, Education, and Welfare, and by grant IM-122 from the American Cancer Society.

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[3]We wish to acknowledge Brenda Cox, Sydney White, and Ann Munson for expert technical assistance, Betty West and Rae Spivey for excellent typing assistance, Dr. I. H. Hopp for assistance with the computerized plots of the data, and Dr. Keith Shelton for his critical evaluation of the manuscript.

[4]In this paper, "activated macrophage" is defined as the population of peritoneal macrophages that exhibit cytostasis and cytotoxicity for neoplastic cells.

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exhibited a single cycle of aberrant cell division in the absence of DNA synthesis.

In this paper, we demonstrate that a population of aneuploid Lewis lung (LL) carcinoma cells in the presence of activated macrophages doubles in number in the absence of detectable DNA synthesis. This "reductive division" leads to a population of tumor cells with an average reduction of 50% in DNA content per cell as determined by the analysis of the DNA content of individual cells by flow microfluorometry (FMF). This aberrant cell division may be related to the lethal events which lead to activated macrophage-mediated tumor cell cytotoxicity.

MATERIALS AND METHODS

Mice, Drugs, Chemicals, and Preparation and Maintenance of Cells

C57BL/6J male mice were obtained from Jackson Memorial Laboratories, Bar Harbor, Me. The culture medium used for maintenance of the LL carcinoma cells and macrophage experiments was Eagle's minimum essential medium with Earle's balanced salt solution, 2× essential amino acids, vitamins, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 20% heat-inactivated fetal bovine serum as previously described (3).

Pyran copolymer, lot No. XA124–177, was obtained from Hercules Chemical Corp (Wilmington, Del) and *C. parvum* was obtained from Burroughs Wellcome Co (Research Triangle Park, NC). For activation of macrophages, mice were injected ip with pyran (25 mg/kg) or *C. parvum* (35–70 mg/kg) 7 days prior to harvest of peritoneal cells. Normal macrophages were obtained from mice that had been untreated or were injected ip with 0.5 ml of a 2.5% solution of oyster glycogen II (Sigma Chemical Co, St. Louis, Mo) 4 days previously.

Peritoneal exudate cells (PEC) were removed from the C57BL/6J mice by washing the peritoneal cavity with Hanks' balanced salt solution as previously described (3,7).

Assay for Cytotoxicity and Preparation of Cells for Analysis of DNA Content by Flow Microfluorometry

Cytotoxicity was assayed morphologically on Lab-Tek slides (Lab-Tek, Naperville, Ill) as previously described (3). When the absolute number of macrophages and/or tumor cells was to be determined, the slides were fixed with methanol at various times after the addition of tumor cells and stained with Wright's stain, and the number of tumor cells and macrophages was determined by a differential count of ten fields each on duplicate slides.

Cultures of peritoneal macrophages and LL cells, or co-cultures of peritoneal macrophages and LL cells, were prepared in 60-mm Petri dishes exactly as previously described for Lab-Tek slides, maintaining the same cell densities (3). Macrophages were removed from Petri plates by treatment with 12 mM lidocaine as previously described (14). LL cells were removed from Petri plates or from monolayers of peritoneal macrophages by mild trypsinization with 0.25% trypsin in EDTA. Cells were washed twice, fixed in 70% cold ethanol, treated with RNase A (10 μg/ml, pH 7.0) for 30 minutes at 37°C, washed, stained with propidium iodide (6.9×10^{-5} M in 3.8×10^{-2} M sodium citrate, pH 7.0), and analyzed by FMF or sorted with a Coulter Electronics TPS-1 as previously described (15,16).

RESULTS

Data from our laboratory have previously demonstrated that activated macrophages inhibit the uptake of ^{125}IUDR into tumor cells as soon as 6 hours after co-cultivation, and are cytotoxic between 24 and 48 hours (7). Microscopic examination by time-lapse cinematography of activated macrophage tumor cell cultures prior to detectable cytotoxicity indicated that the number of tumor cells in the cultures increased prior to detectable cytotoxicity (13). The increase in the number of tumor cells in the presence of activated macrophages without detectable uptake of ^{125}IUDR could not be adequately explained from our current knowledge of the cell cycle. These observations suggested the unique possibility that the tumor cells under a stimulus from activated macrophages divided without previously synthesizing DNA.

LL cells were set up in culture alone, or with activated or normal macrophages, and duplicate cultures were removed at 6, 12, 18, and 30 hours and stained, and the number of tumor cells was determined microscopically in ten high-power fields per culture. During the first 18 hours of culture, there was approximately a doubling of the tumor cells in each group examined: LL alone, LL and activated macrophages (activated macrophages to LL ratios of 15:1), and LL and normal macrophages (normal macrophages to LL ratios of 7:1) (fig 1). After 18 hours, the LL cells alone and the LL cells with normal macrophages continued to increase in parallel, while the LL cells with activated macrophages decreased in number. The similar rate of doubling for the LL cells alone and the LL cells co-cultured

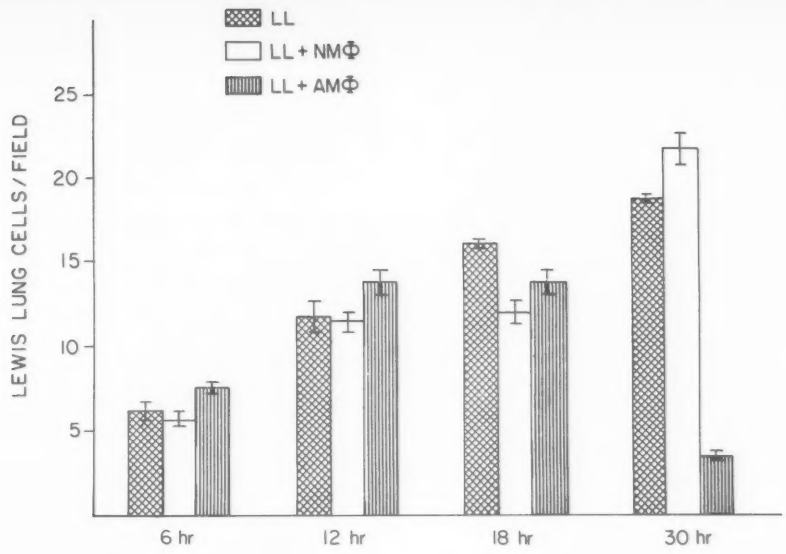

FIGURE 1.—Growth of LL cells alone or in the presence of normal or activated macrophages. LL cells were cultured alone or on monolayers of normal or activated macrophages. Activated macrophages were obtained from the PEC of mice 7 days after ip inoculation with 25 mg/kg of pyran and normal macrophages were obtained from the PEC of mice 4 days after ip inoculation with glycogen. Cultures were removed at various time points, fixed, and stained with Wright's stain, and the number of LL cells was determined on 10 fields of duplicate cultures. Bars = 1 SEM.

with activated macrophages occurred despite a dramatic reduction in ^{125}IUDR incorporation in the LL cells and activated macrophages compared to the LL cells alone (7). These results substantiated the previous cinematographic data (13) and suggested the possibility that LL cells in the presence of activated macrophages divided without incorporating ^{125}IUDR (without synthesizing DNA).

In order to evaluate further this observation, LL cells, activated macrophages, or LL cells incubated with activated macrophages for 12–24 hours and then removed from the macrophage monolayer by trypsinization (but containing contaminating macrophages) were analyzed for DNA content with a flow microfluorometer cell-sorter machine. The activated macrophage population yielded a narrow DNA distribution with a maximum at channel 22 (channel No. = relative fluorescence or DNA content), consistent with a 2C DNA content of about 6.9 pg/cell (fig 2, top panel). This distribution comprised > 96% of the total cell population. The small distribution at channel 44 was probably indicative of a small amount of aggregation, since we have routinely observed between 2% and 4% aggregation with this DNA-staining procedure. The DNA distribution of LL tumor cells is shown in figure 2, center panel. The distribution with a maximum at channel 50 represented the G_1 cells, while the distribution with a maximum at channel 100 represented G_2 + M cells. The S-phase cells were distributed between channels 50 and 100. The G_1 peak of this tumor cell line is near tetraploid (about 4C or 12.8 pg of DNA per cell), as determined from the machine settings used and from calibration with cell lines of known DNA content (data not shown). The DNA distribution analysis of a representative experiment of LL cells co-cultured with activated macrophages for 16 hours is shown in figure 2, bottom panel. There was a prominent distribution at about channel 27 which had the same relative fluorescence as the macrophages alone (ie, approximately 2C DNA content). The distribution of the LL cells, with their greater DNA content, fell between channels 40 and 90, with the maximum of the G_1 cells at about channel 45. Thus, the LL tumor cells were arrested at all cell cycle stages.

Cells with a relative fluorescence of between 20 and 30 were separated by electronic cell sorting from those cells with a relative fluorescence of 45–100 (fig 2, bottom panel). Differential counts indicated that 27% of the cells sorted with a relative fluorescence of 20–30, consistent with a diploid DNA content, were LL cells (table 1). Representative examples of the isolated cells are presented in

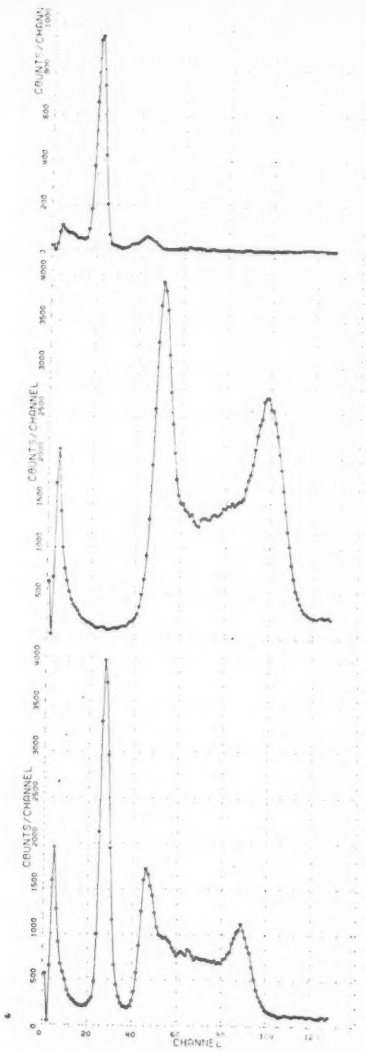

FIGURE 2.—DNA distribution analysis by FMF of activated peritoneal macrophages, LL cells, or LL cells separated from a monolayer of activated macrophages. Peritoneal macrophages from the PEC of mice inoculated ip with 25 mg/kg of pyran 7 days previously were cultured in vitro for 22 hrs, removed from the culture vessels with lidocaine, stained with PI, and analyzed by FMF. LL cells were cultured for 22 hrs, removed from the culture vessels by mild trypsinization, stained with PI, and analyzed by FMF. LL cells were cultured for 16 hrs on a monolayer of pyran activated macrophages, removed by mild trypsinization, stained with PI, and analyzed by FMF. Ordinate: No. of cells, abscissa: channel No. (DNA content). The No. of cells analyzed was 6500 for activated macrophages (top panel), 385,000 for LL (center panel), and 78,000 for LL separated from a monolayer of activated macrophages (bottom panel).

TABLE 1.—Differential counts of LL cells and macrophages sorted by FMF from LL cultures which had been co-cultured with activated macrophages

Relative fluorescence*	% LL cells	% macrophages
20–30	27.0 ± 2.4	73.2 ± 2.5
> 45	97.3 ± 0.33	2.7 ± 0.33

*Cells with a relative fluorescence of 20–30 were separated from cells with a relative fluorescence of > 45. Cytocentrifuge preparations of the sorted cells were stained with Wright's stain and differentials were performed using cultured tumor cells and purified peritoneal macrophages as a reference (mean ± SE, *see* fig 2 for details of experiment).

figure 3. Morphologically, the two cell populations were readily discernible. The LL nuclei had large, though varied, diameters as well as coarse heterochromatin and 3 or more nucleoli (fig 3A). The latter appeared as unstained vesicles in the various fields in figure 3. The cytoplasm of LL cells was not readily discernible in these preparations. The macrophages were much smaller cells with typical nuclear morphology. Frequently, they had azurophilic cytoplasmic granules (fig 3A and B). Moreover, there was a 23% reduction in the diameter of the nuclei from the LL cells in the 2C peak (fig 3B, 7.8 ± 0.13 μm) compared to LL cells in the 4C–8C peaks (fig 3C, 10.2 ± 0.15 μm).

In another experiment, the LL cells removed from activated macrophage monolayers (with contaminating macrophages) were stained for esterase activity prior to DNA distribution analysis. Esterase staining was > 99% positive for the activated macrophage monolayers alone and 0 positive for the LL cells alone. The preparation of LL cells partially purified from activated macrophages was 27% esterase positive. The DNA distribution analysis of the LL cells partially purified from activated macrophages was characterized by a G_1 (2C) peak which represented 41% of the cells in the total distribution analysis. This indicated that 14% of the cells with a DNA content of 2C were not activated macrophages, but presumably were LL cells with a DNA content half their normal tetraploid G_1 value.

To evaluate further our observations, LL cells and co-cultures of LL with normal or activated macrophages were pulsed with colcemid for 2 hours and metaphase chromosomes were prepared. Although numerous cells arrested in metaphase could be detected in the LL and LL co-cultured with normal macrophage monolayers, a > 95% reduction in the number of nuclei with metaphase chromosomes was observed in the LL cells which had been co-cultured with activated macrophages. However, despite the absence of cells in metaphase, numerous

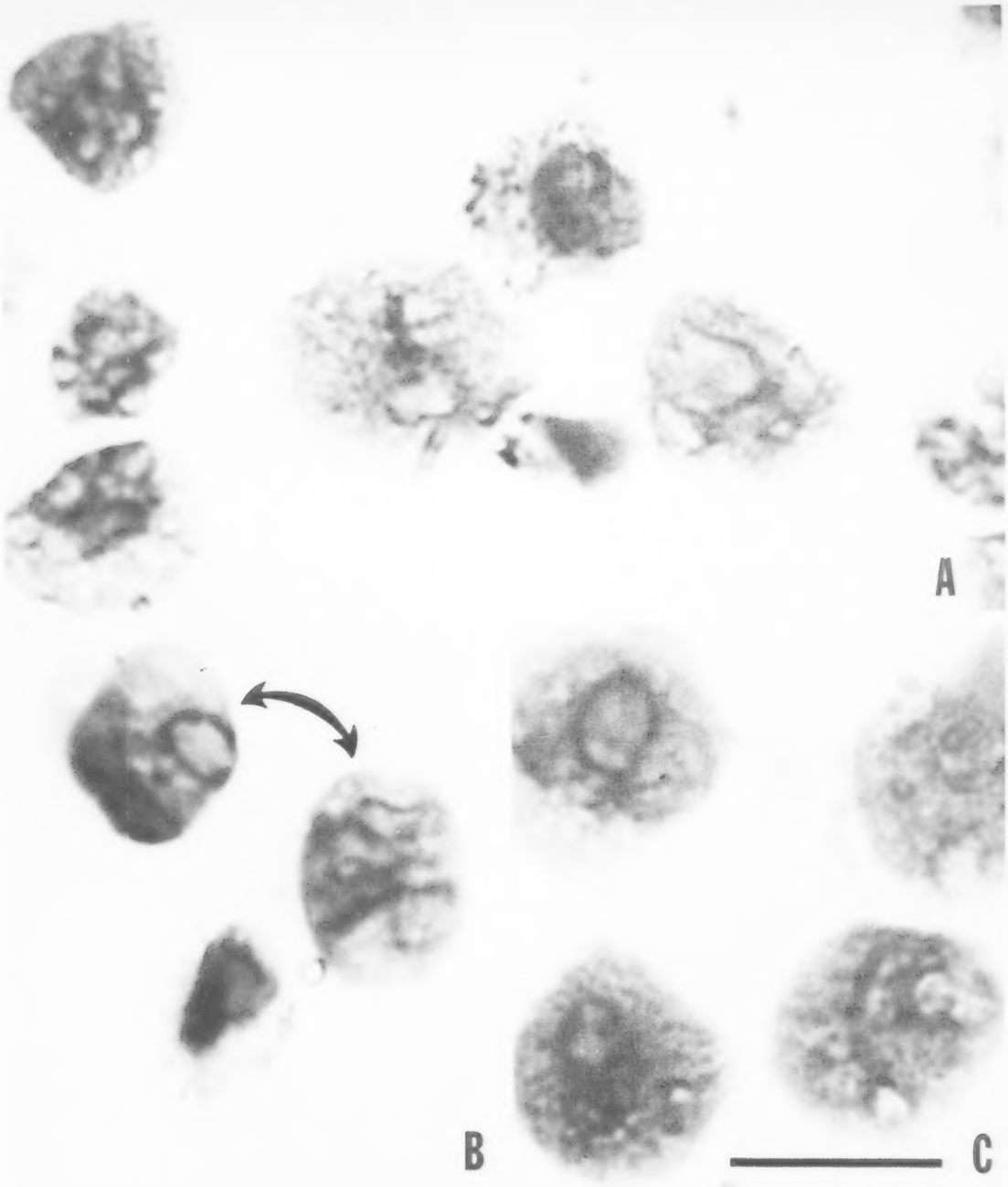

FIGURE 3.—Cytocentrifuge preparations of LL carcinoma cells separated from activated macrophages after 16 hrs of co-culture. The cells had been analyzed by FMF and sorted by the cell sorter. A. LL carcinoma cells and activated macrophages after 16 hrs in co-culture. The activated macrophages are characterized by azurophilic cytoplasmic granules (lysosomes) present in many cells; the nuclei are much smaller than those of the carcinoma cells. B and C. LL cells separated from activated macrophages after 16 hrs in co-culture and then sorted by the cell sorter. B. Cells with a relative fluorescence between 20 and 30; the population is 27% LL carcinoma cells and 73% macrophages. Arrows = LL carcinoma cells. C. Cells with a relative fluorescence > 45; the population is 97% LL cells. Wright's stain; all magnifications are × 3500; dimensional bar = 10 μm.

LL nuclei which appeared to be dividing were detected (data not presented).

DISCUSSION

The present experiments indicate that while activated macrophages inhibited DNA synthesis, there was an increase in the number of LL cells co-cultured with activated macrophages which paralleled that of LL cells alone for the first 18 hours of co-culture (approximately one doubling of cells). To resolve this paradox, the DNA content of LL cells which were co-cultured with activated macrophages for 16 hours was analyzed by FMF. These experiments indicated that subsequent to co-culture with activated macrophages, LL cells with a 50% reduction in their normal DNA content could be detected. Furthermore, after a 2-hour incubation with colcemid, a > 95% reduction in metaphase cells occurred in co-cultures of LL cells and activated macrophages. Moreover, dividing nuclei without chromosome condensation appeared to be a prominent feature of these cultures. The only interpretation consistent with these results appeared to be that the LL cells underwent nuclear and cellular division without synthesizing new DNA. Although this is a novel biologic concept, it is consistent with the ability of activated macrophages to inhibit: (*a*) DNA synthesis, blastogenesis, and lymphoproliferation in mitogen-stimulated lymphocytes (17,18); (*b*) normal mitosis in target tumor cells (12); and (*c*) replication of viruses in mouse embryo fibroblasts (19). This would suggest that the genotypic or phenotypic program for cell division in the tumor cells is such that under these unique circumstances of tumor cell-activated macrophage interaction, cell division can proceed in the absence of: (*a*) DNA synthesis, (*b*) condensation of chromosomes, and (*c*) appearance of the mitotic spindle. Moreover, we suggest that this aberrant division may be related to the lethal event which leads to activated macrophage-mediated tumor cell cytotoxicity.

With respect to the relationship of these findings to current concepts in cell division and multiplication, one must explain the association or linkage of DNA synthesis to subsequent mitosis and cell division. In yeast, Hartwell (20) has demonstrated that DNA replication is a prerequisite for the completion of nuclear division. However, the processes leading to the initiation of nuclear division do not appear to be dependent on prior DNA replication, but at some later stage the apparatus responsible for nuclear division requires duplicated chromosomes. Furthermore, cell separation appears to be dependent either upon DNA replication itself or, more likely, upon the successful completion of nuclear division.

In mammalian cells, however, there is no formal demonstration that the completion of DNA replication actually triggers mitosis. Indeed, after completion of DNA synthesis, all mammalian cells spend a period in G_2 before mitosis. Despite this, the cessation of DNA replication appears to lead to mitosis in most cases. There are, however, numerous exceptions to this rule. Gelfant (21) demonstrated a G_2 arrest in the epidermal cells of the mouse ear. A small percentage of the cells in the renewing epithelium were arrested in G_2, and these were the first cells to enter mitosis when the epithelium was stimulated by wounding. The epithelial cells remained blocked in G_2 indefinitely if the tissue remained undisturbed (22). Similarly, tetraploid cells are common in the liver, heart, and central nervous system (23). Therefore, with respect to the necessity of a DNA content twice that of G_1 as a requirement for nuclear division, the data in the present paper do not disagree with the current dogma for mammalian cells. The LL cells already had a near tetraploid DNA content (ie, almost twice the normal G_1 content) and in the presence of activated macrophage-mediated inhibition of DNA synthesis may still initiate the necessary signals for nuclear division to occur. Therefore, the observation of apparent nuclear division prior to cytokinesis is consistent with the suggestion that cell division requires a completion of nuclear division (20).

The apparent initiation of nuclear division in the absence of detectable normal chromosome condensation, appearance of the mitotic spindle, and detectable metaphase cells is more difficult to explain. The observations of a > 95% inhibition of LL metaphase cells in the presence of activated macrophages, with or without colchicine, are in agreement with recent observations of Krahenbuhl and Remington (12). In the absence of colchicine the reduction of the number of metaphase cells in LL and activated macrophage co-cultures compared to LL and normal macrophages or LL alone could be due to a shortening of the mitotic phase of the cell cycle. What is more puzzling is the inability to detect metaphase cells in the presence of colchicine. This could be due to inhibition of the colchicine-induced effects on the mitotic apparatus by the activated macrophages. Certainly, there is precedent for tumor cells as opposed to normal cells responding differently to various drug and/or regulatory signals. Interestingly, cytochalasin B induces multinucleation in transformed cells while normal cell lines become binucleate (24).

Last, the possibility of karyokinesis and cytokinesis occurring in the absence of chromosome condensation and true mitosis must be considered. This possibility was suggested by the presence of what

appeared to be dividing nuclei in the chromosome preparations of LL and activated macrophage cocultures. Nuclear division in the absence of chromosome condensation and nuclear membrane dissolution, and the formation of a mitotic spindle has been shown to occur in the eukaryotic ameoflagellate *Naegleria fowleri* (25). It can only be speculated that in the present experiments an aberrant contractile ring or some unknown mechanism causes a nuclear division with a relatively random division of chromosomes. However, the distribution of DNA among the daughter cells appears to be markedly even, as opposed to random.

The results in this report suggest an alternative mechanism to lysosomal transfer for macrophage-mediated tumor cell cytotoxicity. Experiments are currently being carried out to determine whether or not this is a general mechanism for macrophage interactions with other neoplastic cells or with normal diploid cells.

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Effect of Maleic Anhydride-Vinyl Ether on the Lymphocyte Subpopulations of Tumor-Bearing, Irradiated Mice

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SUMMARY

The splenic T- and B-lymphocyte populations of BALB/c mice were determined in Madison lung carcinoma 109-bearing animals. Concurrently, some groups of tumored mice were exposed to 500 rads of whole-body irradiation and were treated with one dose of maleic anhydride-vinyl ether (MVE). By direct immunofluorescence, it was found that the percentage of splenic T lymphocytes was significantly depressed in the tumored-irradiated mice. Mitogenic studies revealed that the T lymphocytes were more depressed in the tumored-irradiated mice than in the corresponding nonirradiated tumored mice. MVE was relatively effective in reconstituting the T-cell compartment of these splenic T lymphocytes. The B-cell compartment of the splenic lymphocytes of the tumored-irradiated mice was found to be extremely radiosensitive. Utilizing a specific anti-B serum, no B lymphocytes were detected during the testing. Blastogenic studies using lipopolysaccharide as the mitogenic probe revealed that the incorporation of ^{3}H-TdR of the tumored-irradiated mice was just slightly higher than background values. MVE proved to be relatively ineffective in reconstituting the splenic B cells of the tumored-irradiated mice.

[Cancer Treat Rep 62:1831–1836, 1978]

Lymphocytes are one of the most radiosensitive cell types in the mammalian body (1–3). It has been established that subpopulations of lymphocytes differ in their reactivity towards whole-body irradiation (WBI). In this regard, in vitro studies and studies with irradiated mice have indicated that T cells are more resistant to irradiation than B cells (4–9). It has recently been reported that 500 rads of WBI will cause a rapid and pronounced depletion of normal murine splenic and lymph node cells (10).

However, less is known about the effect of WBI on the T- and B-cell populations of tumor-bearing mice. In the present study, normal BALB/c mice were injected with a syngeneic tumor, Madison lung carcinoma 109. When all of the tumored mice had palpable tumors of ≥ 5 mm in diameter, they were given 500 rads of WBI. Concurrently, some mice were treated with maleic anhydride-vinyl ether (MVE). We report here the T- and B-cell populations of tumored mice receiving 500 rads of WBI, and the effect of MVE on these T and B cells.

MATERIALS AND METHODS

Animals.—BALB/c male mice, 6–8 weeks old, were supplied by the Mammalian Genetics and Animal Production Section, Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md. The animals were housed in plastic cages and fed Purina laboratory chow with water ad libitum. All animals weighed ≥ 23 g before use.

Drug.—MVE was kindly supplied by David Breslow of Hercules Research Center, Wilmington, Del. MVE was dissolved in sterile 0.15 M NaCl, and the pH was adjusted to 7.0 using 1.0 N NaOH. MVE was

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administered ip to the mice at a dose of 25 mg/kg. The tumored mice received only one injection of MVE.

Tumor.—The Madison lung carcinoma 109, derived from a spontaneous neoplasm in a BALB/c mouse, was kindly supplied by Dr. Ruth L. Geran, DTP, DCT, NCI, and has been maintained as a transplantable line in BALB/c mice. For these studies, 5.0×10^5 viable tumor cells were injected sc in the right inguinal region of BALB/c mice. The experimental animals attained palpable tumors of 5–7 mm in size by the 11th day after tumor inoculation. On the 12th day, animals that were to receive WBI were randomly distributed to the appropriate groups and were irradiated and injected ip with MVE within ½ hour. The day of irradiation and/or drug treatment was considered Day 0 and the in vitro assays were started 1 day later.

Whole-body irradiation of mice.—BALB/c mice in lucite boxes were exposed to X-rays using a General Electric Maxitron 300 instrument operated at 300 KVp and 20 mA. The beam was filtered through an inherent filter of 2 mm copper, measured at 1.54 mm copper, with an effective energy of 96 KeV. The midline dose rate was approximately 40 rads/minute.

Lymphocyte preparation.—Lymphocytes were obtained from BALB/c mice by aseptic removal of the spleens. The spleens were triturated in 10 ml of sterile RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 1% penicillin-streptomycin in sterile 15 × 150-mm Petri dishes (Falcon Plastics, Oxnard, Calif). The dishes were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 1 hour in order to remove adherent macrophages. The nonadherent cells were collected and incubated at room temperature for approximately 5 minutes with 0.11 M ammonium chloride to deplete rbcs. The nonadherent, rbc-depleted cells, taken up in 4 ml of RPMI-1640, were layered over 5 ml of Ficoll-Hypaque separation medium containing 12 parts of 14% Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, NJ) and five parts of 32.8% Hypaque sodium (diatrizoate sodium, Sterling Winthrop Research Institute, Rensselaer, NY; specific gravity, 1.09). The gradient was centrifuged at 300 × *g* for 30 minutes at room temperature. The interface containing the separated lymphocytes was collected, washed three times with RPMI-1640, checked for viability with trypan blue, and brought to a concentration of 3.3×10^6 viable cells/ml.

Antisera.—Mouse anti-T serum was produced in adult male New Zealand white rabbits (National Institutes of Health). The brains of five C57BL/6 mice were removed; 2.0 ml of phosphate-buffered saline and 2.5 ml of complete Freund's adjuvant were added to a Virtis homogenizer and emulsified. Each of three rabbits was injected sc with 1.0 ml of the emulsion into four sites in its back. Subsequent booster inoculations every other week were similarly administered with the emulsion made up in incomplete Freund's adjuvant. The antiserum used in these reported experiments was from a pool of three rabbits that received an initial injection and five booster injections. Ten ml of rabbit antimouse brain serum was absorbed with 4 ml of packed, washed BALB/c liver cells at 37°C for 30 minutes and at 4°C overnight. The next day, the BALB/c liver-absorbed antiserum was absorbed in a similar fashion with 4 ml of washed BALB/c rbcs. The absorbed antiserum was heat-inactivated for 30 minutes at 56°C and 0.5-ml samples were stored at –40°C. Mouse anti-immunoglobulin serum was produced in New Zealand white rabbits. Polyvalent mouse IgG (Litton Bionetics, Kensington, Md) was digested with pepsin (11). The $F(ab')_2$ fragment was isolated on a Sephadex G-150 column. A molecular weight of 102,000 was determined by SDS acrylamide gel electrophoresis (12) for the $F(ab')_2$ peak. Five mg of $F(ab')_2$ was emulsified with complete Freund's adjuvant and inoculated sc into each of three rabbits as above. Each rabbit received approximately 1 mg of mouse $F(ab')_2$ per injection. The rabbits were administered four additional sc booster inoculations in incomplete Freund's adjuvant. The anti-$F(ab')_2$ serum was extensively absorbed against BALB/c liver and rbcs as above. The antiserum was heat-inactivated as above and stored in 0.5-ml volumes at –40°C. The specificity was determined in a mitogen assay using a Sepharose 4B-rabbit antimouse $F(ab')_2$ affinity column (13) to separate T and B cells. The titer and specificity of the anti-T and -B sera are shown in table 1. Goat antirabbit IgG labeled with fluorescein isothiocyanate (lot No. 2122801) was obtained from the Division of Cancer Cause and Prevention, NCI. The optimal dilution for its use in the direct immunofluorescent studies was determined to be 1:20.

Response to mitogens.—Viable, nonadherent, rbc-depleted lymphocytes (5.0×10^5) contained in 150 μl were added to each well of a Micro-test II Tissue Culture Plate (3040, Falcon Plastics). Lipopolysaccharide W (Difco, Detroit, Mich [10 μg/ml]) or concanavalin A (Con A) (Pharmacia Fine Chemicals [5 μg/ml]) contained in 50 μl was also added. These concentrations were found to be optimal by wide-range titrations of the various mitogens. Cultures were incubated for 48 hours at 37°C in a 5% CO_2 and 95% humidified air atmosphere, and 0.5 microcurie of ^{3}H-methylthymidine (NET-027, specific activity, 6.7 curies/mmol; New England Nuclear Corp,

TABLE 1.—Titer and specificity of the anti-T and -B sera used as 1st antibodies in the indirect immunofluorescent studies

	Splenic lymphocytes from nu/nu mice (%)	BALB/c thymic lymphocytes (%)
Rabbit antimouse brain serum (T)		
Indirect immunofluorescence (1:100)	11	94
Specific ^{51}Cr release (1:128)	6	100

Rabbit antimouse $F(ab')_2$ serum (B)

Indirect immunofluorescence utilizing rabbit antimouse $F(ab')_2$ serum coupled to Sephadex G-200 to separate BALB/c splenic T and B lymphocytes

	Unseparated lymphocytes (%)	T lymphocytes (%)	B lymphocytes (%)
Anti-T (1:75)	48	93	6
Anti-B (1:75)	31	3	93
Mitogenic studies utilizing $F(ab')_2$ serum coupled to Sephadex G-200 as above to separate T and B lymphocytes			
Con A	49.2*	83.2	1.2
Phytohemagglutinin	25.6	63.5	2.3
LPS	17.3	2.3	24.8

*Stimulation ratio: mean of 3 cultures mitogen added minus mean of 3 cultures no mitogen added.

Boston, Mass) in 50 μl was added to each well. The cultures were incubated for an additional 18–24 hours. The cells were washed with deionized water by use of a Mash II cell harvester (Microbiological Associates, Bethesda, Md). All radioactive filters were counted in a liquid scintillation spectrometer (Packard Model 3385 Tri Carb, Downer's Grove, Ill) with Aquasol (New England Nuclear Corp) as the scintillation fluid. The results of three cultures are expressed as mean cpm, ie, mean of three cultures mitogen added minus mean of three cultures no mitogen added.

RESULTS

Tumor development.—The growth of the Madison lung carcinoma 109 in the various groups of treated mice is shown in table 2. In general, irradiation or MVE treatment resulted in smaller tumors, but a significant regression of tumor size was detected only for Day 11 in the group treated with MVE and radiation.

Immunofluorescence.—For all indirect immunofluorescent studies, lymphocytes of the various groups of tumored mice were isolated from the spleen, purified by Ficoll-Hypaque gradient centrifugation, and reacted with either the anti-T or -B serum. The relative percentage of T cells in the groups of mice is shown in table 3. The tumored mice that received 500 rads of WBI have a significantly reduced percentage of T cells. On the other hand, the tumored-irradiated mice that received pyran had a T-cell percentage significantly higher than the tumored-irradiated group. The tumored-nonirradiated mice treated with MVE usually had slightly higher T-cell percentages than the tumored-control group. The B-cell percentages for the various groups of treated mice are shown in table 4. It was found that 500 rads of WBI immediately and totally depleted lymphocytes carrying immunoglobulin surface markers.

TABLE 2.—Determination of tumor size of Madison lung carcinoma 109-bearing mice and tumored-irradiated mice treated with MVE

Time (days)	Tumored control (mm)	500 rads alone (mm)	*P* value	500 rads + MVE (mm)	*P* value	MVE alone (mm)	*P* value
1	8.8 ± 0.4*	10.0 ± 0.5	< 0.10†	9.8 ± 0.2	< 0.05	8.5 ± 0.7	< 0.10
4	12.1 ± 0.6	11.4 ± 0.7	< 0.10	11.2 ± 0.1	< 0.10	10.2 ± 0.8	< 0.10
7	14.3 ± 0.6	13.4 ± 0.7	< 0.10	13.5 ± 0.2	< 0.10	14.8 ± 1.2	< 0.10
11	17.8 ± 0.6	Not determined	—	15.5 ± 0.2	< 0.02	16.9 ± 1.4	< 0.10

*Values are mean ± SE; No. = 10.

†Probability is calculated vs tumor control for each determination made (Student's t test).

TABLE 3.—Percentage of positive fluorescence of splenic T lymphocytes from the various groups of tumored-irradiated mice

Time (days)	Experimental control	Tumored control	500 rads alone	500 rads + MVE	MVE alone
1	24.7 ± 0.3*	25.0 ± 1.2	17.7 ± 0.3	23.0 ± 0.6	28.0 ± 3.1
4	24.6 ± 2.1	23.0 ± 1.7	11.3 ± 0.3	18.6 ± 0.6	24.3 ± 2.6
7	24.7 ± 0.9	29.0 ± 2.7	13.3 ± 1.3	21.3 ± 0.7	27.0 ± 1.0
9	23.4 ± 0.3	25.0 ± 0.6	15.0 ± 1.0	16.2 ± 0.9	26.7 ± 1.9
11	22.3 ± 0.3	22.0 ± 2.1	Not done	25.2 ± 2.1	20.3 ± 2.0

*Values are mean ± SE of ≥ 100 randomly enumerated lymphocytes (3 readings).

TABLE 4.—Percentage of positive fluorescence of splenic B lymphocytes from the various groups of tumored-irradiated mice

Time (days)	Experimental control	Tumored control	500 rads alone	500 rads + MVE	MVE alone
1	45.3 ± 0.7*	43.0 ± 1.0	0	0	46.0 ± 1.5
4	47.7 ± 1.5	49.0 ± 1.2	0	0	58.0 ± 1.0
7	45.7 ± 1.9	57.0 ± 1.2	0	0	58.7 ± 0.7
9	47.0 ± 1.0	55.0 ± 1.0	0	0	49.0 ± 1.2
11	44.7 ± 0.7	48.7 ± 1.3	Not done	0	58.6 ± 0.6

*Values are mean ± SE of ≥ 100 randomly enumerated lymphocytes (3 readings).

MVE failed to reconstitute the B-cell population of the tumored-irradiated mice.

Lymphocyte proliferative response.—Blastogenic studies were carried out with the nonadherent rbc-depleted splenic lymphocytes of the various groups of tumored mice. The Con A reactivity of these mice is shown in table 5. The tumored group treated with MVE and the nontumored-control group were the only groups that incorporated larger amounts of ^{3}H-TdR than the tumored-control group. The irradiated mice, untreated or treated with MVE, incorporated significantly smaller amounts of ^{3}H-TdR than the tumored-control group. The mitogenic responses of the B lymphocytes to lipopolysaccharide (LPS) reveal that the irradiated mice, untreated or treated with MVE, were virtually unreactive to LPS. On the other hand, the nonirradiated-tumored mice treated with MVE usually incorporated significantly higher amounts of ^{3}H-TdR than the tumored controls.

DISCUSSION

It has been established that normal T and B lymphocytes differ in their sensitivity to WBI (4–8). Prosser (9) has shown that human T lymphocytes treated in vitro with 200–1000 rads of radiation are between two and five times more resistant than B cells. Kwan and Norman (14) have followed the in vitro survival of human peripheral blood lymphocytes and thymocytes measured 4 days after graded doses of radiation. They found considerable heterogeneity among lymphocytic subpopulations with respect to radiosensitivity. B cells appeared to be slightly more radiosensitive than T cells. Katoaka and Sado (15) have found that in normal C3H mice the splenic T lymphocytes were more resistant to radiation than splenic B cells. Additionally, they found a subpopulation of T cells, estimated to be approximately 8% of the T lymphocytes in the spleen, to be highly radioresistant. Rozing et al (16) have found that a dose of 3 microcuries of ^{89}Sr/g of body weight causes a depletion of B cells in normal (C57BL × DBA)F_1 mice. Their data also suggest that the bone marrow is involved in the generation of the B-cell population after irradiation and reconstitution. Kettman and Mathews (17) have reported that sheep rbc-primed spleen cells of normal (C57BL × DBA)F_1 mice transferred into the footpad of irradiated normal recipients will cause no delayed-type footpad swelling response. From these data the authors suggest that there are two components necessary for the transfer of delayed hypersensitivity; one component is the radioresistant T cell contributed by the donor and the second component is the radiosensitive T cell derived from the host. Recently, Anderson et al (10) found that normal CBA/J mice exposed to 500 rads of WBI had a rapid and severe depletion of B cells in the spleen and lymph nodes.

However, the T- and B-lymphocyte populations of tumor-bearing animals subjected to WBI have received less attention. Gorczynski (18,19) and Gorczynski and Norbury (20) have shown that spleens of murine sarcoma virus-regressor BALB/c mice contain T lymphocytes capable of protecting sublethally irradiated mice from the lethal effect of

TABLE 5.—Proliferative response of splenic lymphocytes from the various groups of tumored-irradiated mice*

Mitogen	Experimental control	Tumored control	500 rads alone	500 rads + MVE	MVE alone
			Day 1		
Con A	128,439†	140,231	84,534	135,117	152,467
LPS	13,795	12,934	293	212	17,757
			Day 4		
Con A	144,969	136,045	48,275	30,164	123,875
LPS	14,917	17,789	231	−23	41,334
			Day 7		
Con A	159,502	103,077	60,879	80,976	142,561
LPS	13,101	34,850	455	458	41,228
			Day 9		
Con A	1.3,817	122,265	55,779	7303	152,467
LPS	14,014	30,302	165	−68	17,757
			Day 11		
Con A	129,925	65,609	Not done	13,262	148,721
LPS	12,524	17,265	Not done	122	39,416

*5×10^5 nonadherent, rbc-depleted, splenic lymphocytes incubated at 37°C for 48 hrs in the presence of 5 μg/ml of Con A or 20 μg/ml of LPS. 0.5 microcurie of ^{3}H-TdR was added and then reincubated for an additional 18 hrs.

†Mean cpm; *see* Materials and Methods.

murine sarcoma virus inoculation. In the present study, BALB/c mice were injected with a syngeneic tumor, Madison lung carcinoma 109, and were subjected to 500 rads of WBI. The tumors of the group of mice that received WBI did not significantly differ in size from those of the tumored-control group (table 2). On the other hand, the T cells of the irradiated-tumored mice, as detected by indirect immunofluorescence, had T-cell percentages significantly lower than the nonirradiated-tumored mice during the study (table 3). Further T-cell reactivity of the irradiated-tumored mice was investigated utilizing Con A as a mitogenic probe in lymphocyte proliferative studies. It was found that splenic lymphocytes from irradiated-tumored mice reacted with Con A had a decrease of from 1.6-fold (Day 1) to 2.8-fold (Day 4) in the incorporation of ^{3}H-TdR when compared to nonirradiated-tumored mice (table 5). Both the immunofluorescent and mitogenic studies indicate that although T cells are relatively radioresistant, there is a significant decrease in the percentage of T cells bearing the θ antigen, and a significant decrease in the incorporation of ^{3}H-TdR in tumored mice receiving 500 rads of WBI.

The total depletion of B cells in the tumored-irradiated mice (table 4) was unexpected. One day after giving 500 rads of WBI (as well as throughout the duration of the experiment), we were unable to detect any positively stained B cells. The possibility that our anti-F(ab')$_2$ serum was not detecting IgG-positive splenic lymphocytes of the tumored-irradiated mice can be ruled out by the specificity of this antiserum (table 1), in addition to the fact that the B-cell percentages detected for the normal controls are within the values reported for normal BALB/c mice (21). The data of Anderson et al (10) clearly indicate that a large dose (500 rads) of WBI will cause a severe but not total depletion of the B cells of normal female CBA mice. They found that the splenic lymphocytes of the normal irradiated mice contained approximately 10% B cells for up to 5 days after irradiation, and nearly 20% splenic B cells up to 9 days after irradiation. The apparent discrepancy between these two studies could be attributed to either strain or sex differences of the mice utilized, or, in the present study, the tumor-bearing mice that were used for the radiation studies. Radiation exposure conditions were similar in both studies. The present lymphocyte proliferative studies with LPS (table 6) also reveal a drastic reduction of the incorporation of ^{3}H-TdR in the B cells of the tumored-irradiated mice. The corrected values for the incorporation of ^{3}H-TdR (ie, mean cpm) ranged from about 150 cpm (Day 9) to 450 cpm (Day 7). The incorporation of ^{3}H-TdR in the same time periods for the tumored-nonirradiated mice ranged from 30,300 to 34,850 cpm respectively. These data would also indicate that the splenic B-cell population of the tumored irradiated mice is practically nonexistent, about 1% of the incorporation of the tumored-nonirradiated mice being detected throughout the study.

It has been shown that MVE possesses potent immunostimulatory activity (22–25). Baird et al (26)

have reported that spleen cells from normal mice administered MVE showed 25–40 times greater incorporation of ^{3}H-TdR in the absence of mitogen when compared to cells from untreated mice. We have recently shown that MVE appears to reconstitute the T-cell population and has a transitory stimulatory effect on the splenic B-cell population of tumor-bearing mice. In the present study, the immunofluorescent data (table 4) indicate MVE to be relatively ineffective in reconstituting the percentage of B cells from the spleens of tumored-irradiated mice. On the other hand, MVE treatment of the tumored-irradiated mice resulted in significantly elevated percentages of splenic T lymphocytes during the first week of observations and a slight increase in T cells for the subsequent observation periods. The mitogenic data (table 5) reveal that the T lymphocytes from the tumored-irradiated mice treated with MVE did not consistently cause an increase in the incorporation of ^{3}H-TdR over values detected for the lymphocytes of the non-MVE-treated tumored-irradiated mice. The Con A-sensitive T cells of the MVE-treated tumored-irradiated mice did, at times (Days 1 and 7), result in significantly higher increases in the incorporation of ^{3}H-TdR than those detected for the corresponding non-MVE-treated mice. MVE treatment had less effect in increasing the incorporation of ^{3}H-TdR of the LPS-sensitive cells in the tumored-irradiated mice. Only on Day 7 did the MVE-treated mice incorporate ^{3}H-TdR at levels comparable to the non-MVE-treated mice. The mitogenic data reveal that MVE's effect on the T and B lymphocytes of tumored-irradiated mice is not static. There is a definite biphasic response of the lymphocytes from the MVE-treated mice to Con A and LPS.

The data presented in this study show that as a nonspecific immunostimulator, MVE is not as effective in stimulating either T or B lymphocytes under conditions in which the experimental mice are carrying a syngeneic tumor and subsequently exposed to WBI. Compared to the effects in nonirradiated-tumored mice, MVE appeared to stimulate T cells in irradiated animals as shown by the immunofluorescent data, but lymphocyte stimulation was not shown by the mitogenic data. B-cell population was so extensively depleted by 500 rads of WBI that the ability of MVE to reconstitute the B-cell compartment under these conditions would be minimal.

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DIVEMA-Methotrexate: Immune-Adjuvant Role of Polymeric Carriers Linked to Antitumor Agents

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SUMMARY

The folate antagonist methotrexate (MTX) was linked covalently to the divinylether-maleic anhydride (DIVEMA) copolymer yielding a copolymer (DIVEMA-MTX) with an MTX content of 25.2%. Antitumor evaluation of DIVEMA-MTX in mice has shown a potentiated activity of the copolymer over either component or the combination of DIVEMA + MTX against L1210 leukemia and Lewis lung carcinoma at optimal equivalent doses and schedules for MTX. In addition to a sustained plasma pharmacokinetic effect of MTX, an adjuvant effect of the DIVEMA polymer was shown to contribute to the enhanced antitumor activity. A test for the relative immune competence of DIVEMA-MTX is its protective action in combination with an L1210 tumor cell vaccine in mice challenged with live L1210 cells. DIVEMA-MTX was still potent as an immune-adjuvant in this experiment but its effect was slightly reduced compared to equivalent doses of DIVEMA alone or the combination of DIVEMA + MTX (60% versus 90% survivors). DIVEMA or DIVEMA-MTX used as an adjuvant in conjunction with a suboptimal treatment course of MTX increased the lifespan of L1210-bearing mice relative to treatment with MTX alone. However, the antitumor effect obtained was lower than with an optimal treatment of DIVEMA-MTX or MTX. Peritoneal macrophages from mice treated with 10 mg/kg of DIVEMA-MTX or DIVEMA had similar but slightly lower cytostatic effects towards M109 lung carcinoma cells in vitro. In contrast to DIVEMA, the macrophage-mediated cytostasis of DIVEMA-MTX was strongly dose-dependent and diminished at 20 mg/kg. These results indicate that the immune-potentiating effect of DIVEMA can be maintained in the DIVEMA-MTX complex and thus can be integrated as a specific property in the design of polymer-linked antitumor agents.

[Cancer Treat Rep 62:1837–1843, 1978]

The biologic activity of the divinylether-maleic anhydride copolymer (DIVEMA, pyran) has been intensively studied (1). DIVEMA is an agent capable of strongly modulating the host's immune response. It is a potent interferon inducer (2) and has been shown to possess antitumor activity (3,4). Moreover, DIVEMA has shown potent enhancement of the antitumor activity against both a murine leukemia and solid tumor when it is used as an adjuvant in conjunction with alkylating cytotoxic agents (5). The cytotoxic effect of DIVEMA is most likely due to the specific activation of macrophages, which has been demonstrated in several studies (6,7). Macrophages stimulated by DIVEMA were found to specifically infiltrate the tumor site in M109 lung carcinoma-bearing mice and to inhibit tumor cell DNA synthesis (8).

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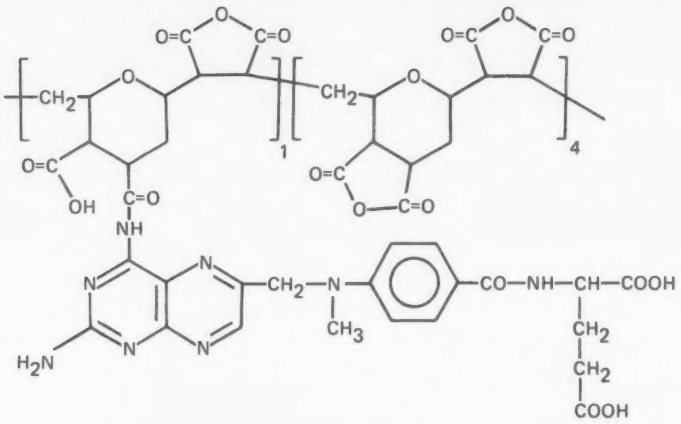

FIGURE 1.—Structure of DIVEMA-MTX, anhydride form. The sites of fixation depicted represent only one of the possible linkages with respect to the maleic anhydride groups of DIVEMA and the amino groups of the pteridinyl moiety of MTX.

As a part of a program on the development and investigation of antitumor agents linked to synthetic polymeric carriers (9), we have synthesized a copolymer of DIVEMA and methotrexate (MTX) by fixation of MTX at the amino groups of the pteridinyl moiety to the maleic anhydride groups of DIVEMA (10). The structure of the DIVEMA-MTX copolymer (NSC-282447) is shown in figure 1. DIVEMA-MTX has antitumor activity against L1210 leukemia and Lewis lung carcinoma which is greater than that of either MTX or DIVEMA alone or the combination of DIVEMA + MTX using equivalent optimal doses and schedules for MTX in systemic treatment (11). In pharmacokinetic studies, a sustained plasma concentration of MTX was found, and this suggested one reason for the enhanced antitumor activity of the DIVEMA-MTX copolymer. However, an increased antitumor effect against L1210 leukemia, relative to MTX alone, was also observed when mice were treated with low, suboptimal doses of the DIVEMA + MTX combination, which suggests an adjuvant action of DIVEMA.

The immune-adjuvant role of DIVEMA when used as a carrier substituted partially with an immunosuppressive antitumor drug has been of particular interest to us because it might provide a potential beneficial effect in addition to the pharmacologic effects aimed for by polymer-linked antitumor agents. In the present study, we therefore compared the relative immune competence of DIVEMA-MTX and DIVEMA, using an adjuvant schedule in the systemic treatment of L1210 leukemia with MTX. We also investigated the relative cytotoxic capabilities of macrophages stimulated by DIVEMA or DIVEMA-MTX toward M109 carcinoma cells. Furthermore, the established protecting action of DIVEMA given in combination with L1210 tumor cell vaccine (12), to challenge with live L1210 cells, was used as a test specifically to assess whether DIVEMA's immune-potentiating effect was retained in the DIVEMA-MTX copolymer. When the L1210 vaccine was administered to mice in a suboptimal manner inducing only minimal antitumor immunity, DIVEMA was found to be the most potent of a variety of biologic and chemical immune-adjuvant agents (13).

MATERIALS AND METHODS

Mice

Male CD2F$_1$ (BALB/c × DBA/2) and C57BL/6 mice, 6–8 weeks old, were obtained from the Mammalian Genetics and Animal Production Section, Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI) (Bethesda, Md). They were housed in plastic cages with Purina laboratory chow and tap water ad libitum; they weighed at least 23 g before being used in experiments.

Drugs

MTX was obtained from the DTP, DCT, NCI, and was dissolved in 2% $NaHCO_3$. Samples of DIVEMA

copolymer with various molecular weights were supplied by Dr. D. S. Breslow, Hercules Inc (Wilmington, Del). Besides the clinically used DIVEMA (NSC-46015) (average molecular weight, 22,000), a polymer identical to that used for the synthesis of the DIVEMA-MTX copolymer was employed in this study (lot. No. X20706–45, Hercules Inc). It had a similar average molecular weight of approximately 24,000 but a narrower molecular weight distribution than NSC-46015.

The synthesis of the DIVEMA-MTX copolymer has been described previously (10). The polymer was purified by solvent extraction and multifold re-precipitation, and characterized, in the poly-anhydride form, by thin-layer chromatography, infrared spectroscopy, ultraviolet spectroscopy, and elemental analysis. It had an MTX content of 25.2% as determined spectroscopically at λ_{max} = 304 nm (10).

Both DIVEMA and DIVEMA-MTX were dissolved in either 2% $NaHCO_3$ (pH 8.3) or 0.85% NaCl with the addition of 0.1 M NaOH to a final pH adjustment of 7.5. DIVEMA-MTX was reconstituted immediately prior to use since hydrolysis to free MTX was suggested from previous experiments. At the pH used, DIVEMA and DIVEMA-MTX are, under hydrolysis of the maleic anhydride groups, quantitatively converted to the polyanionic, carboxylate form (10).

Tumors

The M109 lung carcinoma, which arose spontaneously in a BALB/c mouse in 1964, was received from Dr. Ruth I. Geran (DTP, DCT, NCI). Cell strains derived from the M109 carcinoma were established and maintained in Roswell Park Memorial Institute Medium 1640 supplemented with 20% heat-inactivated (56°C for 30 minutes) fetal calf serum, gentamicin (100 μg/ml), 0.075% $NaHCO_3$, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (RPMI-FCS). The L1210 lymphoid leukemia was maintained and continuously passaged ip in female DBA/2 mice, according to standard protocols of the DTP, DCT, NCI (14). For chemotherapy experiments, 0.1 ml of ascites fluid diluted 1:1000 from donor mice was injected ip into male $CD2F_1$ mice at an inoculum size of 10^5 cells.

Preparation of L1210 Vaccine

L1210 cells used for vaccine preparation were adjusted to a concentration of 5×10^7 cells/ml in Eagle's minimal essential medium with 100 units of penicillin/ml and 100 μg of streptomycin/ml. They were exposed to 5000 rads of radiation from a double-tube Westinghouse Quadrocondex X-ray machine and administered ip to $CD2F_1$ mice at an injection volume of 0.2 ml. Seven days later, mice were challenged by ip injection of 1×10^4 live L1210 cells. Animals receiving both tumor cell vaccine and adjuvant were administered the drug ip at 0.01 ml/g 1–2 hours after receiving vaccine. Control groups showed that systemic leukemia was never produced by administration of the vaccine alone.

Preparation of Macrophage Cultures

Nonactivated BALB/c or C57BL/6 mouse peritoneal macrophages were prepared as described previously (15). Exudates from five mice in each group were collected 6 days after drug treatment, pooled, washed in 20 ml of Hanks' balanced salt solution, and resuspended in 10 ml of RPMI-FCS. Peritoneal macrophages were purified by adherence on 10 × 20-mm tissue culture dishes. After 1 hour of incubation at 37°C, the adhering cells were gently scraped off into RPMI-FCS, adjusted to 1×10^6 viable cells/ml, and kept in an ice bath before use. More than 95% of the cells prepared in this manner had morphologic characteristics of macrophages and the capability to ingest latex beads.

Assay for Macrophage-Mediated Cytostasis

The ability of activated macrophages to diminish M109 target cell proliferation was measured by the DNA synthesis inhibition assay as described previously (16). Target cells were trypsinized from exponentially growing cultures and resuspended at 5×10^4 cells/ml of RPMI-FCS, and 2-ml aliquots were placed in 30-mm tissue culture dishes. Purified peritoneal macrophages (1.0 ml [1.0×10^6 cells/ml]) were added to the target cell cultures. DNA synthesis of the target cells was assessed after 20 hours of incubation at 37°C by determination of ^{3}H-thymidine incorporation. Triplicate cultures of each set of dishes as well as cultures consisting of tumor cells alone and macrophages alone were pulsed with 2.0 microcuries of ^{3}H-thymidine (specific activity, 10 curies/mmol) for 2 hours at 37°C. Cells were then detached with trypsin, centrifuged at 600 × *g* for 10 minutes, and resuspended in 0.5 ml of Dulbecco's phosphate-buffered saline. After addition of 4 ml of ice-cold 10% trichloroacetic acid to each tube, DNA was precipitated for 30 minutes at 4°C. The resultant precipitate was collected on glass-fiber paper (Millipore Corp, Bedford, Mass) and washed with cold 10% trichloroacetic acid. Filters were air-dried and assayed for ^{3}H content in a Packard Tri-Carb scintillation counter with Aquasol solubilizer (New

England Nuclear Corp, Boston, Mass). The percentage of specific inhibition of DNA synthesis was calculated from the formula:

$$\% \text{ specific inhibition} = \frac{(cpm_N - cpm_E)}{cpm_E} \times 100$$

where cpm_N = mean cpm in cultures containing effector cells from normal control mice and cpm_E = mean cpm in cultures containing test effector cells. Under the culture conditions used, macrophages do not proliferate and incorporate a significant amount of thymidine (16).

RESULTS

Adjuvant Effect of DIVEMA and DIVEMA-MTX Against Murine L1210 Leukemia

In previous chemotherapy experiments, we have shown increased antitumor activity of the DIVEMA-MTX copolymer against L1210 leukemia in mice, relative to treatment with MTX. An intermittent treatment schedule, every fourth day × 5, gave a maximum increase in lifespan (ILS) for DIVEMA-MTX of 205% at a dose of 8 mg/kg/day (MTX equivalents), with 20%–30% long-term survivors. In comparison, MTX at a dose of 20 mg/kg/day gave 165% ILS (11). When mice were treated with equivalent doses of the combination of DIVEMA + MTX, the maximum ILS did not exceed that obtained with MTX alone. However, low, suboptimal doses of the combination of DIVEMA + MTX still produced a significant increase in survival time compared to MTX, 95% versus 46% ILS at 5 mg/kg/day. DIVEMA alone is ineffective against L1210. To further evaluate the relative adjuvant effect due to the DIVEMA polymer, L1210 leukemia-bearing mice were treated with a single dose of the polymer in conjunction with an intermittent treatment course with MTX. In this experiment, the antitumor effect of MTX (20 mg/kg) decreased dramatically when treatment was omitted on Day 5 of an every fourth day × 5 schedule, while a single dose of DIVEMA given in place of MTX on Day 5 led to a relative increase in survival by > 50% (table 1). Adjuvant treatment with either the DIVEMA-MTX copolymer or the combination of DIVEMA + MTX in the same manner was also effective in increasing the lifespan of mice. However, the effect of DIVEMA-MTX was lower than that attained in systemic, intermittent treatment, and no long-term survivors were observed.

Potentiation of L1210 Vaccine by DIVEMA and DIVEMA-MTX to L1210 Tumor Cell Challenge

Previous studies established that DIVEMA, when administered concomitantly (within 2 hours) with radiation-inactivated L1210 vaccine 7 days prior to challenge with live L1210 cells, greatly potentiated the resistance of mice to the tumor challenge (13). Single ip doses of 5–20 mg/kg of DIVEMA produced almost complete protection of mice against subsequent tumor inoculation. Therefore, this test system was thought to be of particular significance in assessing whether (*a*) full or partial immune competence would be maintained for the DIVEMA-MTX copolymer, and (*b*) the co-administration of the

TABLE 1.—Adjuvant effects of DIVEMA and DIVEMA-MTX in the treatment of L1210 leukemia compared with MTX*

Drugs	Dose† (mg/kg/day) DIVEMA	Dose† (mg/kg/day) MTX	Schedule (days)	MST‡ (days)	ILS (%)	S/T§
Control	—	—		8.5	—	0/10
MTX	—	20	1, 9, 13, 17	13.0	47	0/10
MTX	—	20	1, 5, 9, 13, 17	22.0	159	0/10
MTX		20	1, 9, 13, 17	16.8	104	0/10
DIVEMA	23.4		5			
MTX		20	1, 9, 13, 17	21.5	153	0/10
DIVEMA-MTX	23.4	8	5			
MTX		20	1, 9, 13, 17	20.8	143	0/10
DIVEMA + MTX	23.4 +	8	5			

*Male CDF_1 mice were inoculated with 1×10^5 L1210 cells ip on Day 0 and treated ip on Day 1, q4d × 5, or until death.
†Dose of DIVEMA-MTX expressed as DIVEMA and MTX equivalents.
‡MST = median survival time.
§S/T = 30-day survivors/total.

TABLE 2.—Potentiation of L1210 vaccine by DIVEMA and DIVEMA-MTX

Treatment group No.	Vaccine (1×10^7 cells)*	Drug†	Dose (mg/kg)‡ DIVEMA		MTX	L1210 challenge (1×10^4 cells)	Results§ MST (days)	S/T
1	Day –7	—				—	> 90	10/10
2	Day –7	—				Day 0	11	0/10
3	—	—				Day 0	9	0/10
4	—	—				—	> 60	10/10
5	Day –7	DIVEMA¶,**	20		—	Day 0	> 60	10/10
6	Day –7	DIVEMA¶,††	20		—	Day 0	> 60	8/10
7	Day –7	DIVEMA**,‡‡	20		—	Day 0	> 60	9/10
8	Day –7	DIVEMA††,‡‡	20		—	Day 0	> 60	9/10
9	Day –7	DIVEMA‡‡ + MTX**	20	+	6.8	Day 0	> 60	9/10
10	Day –7	DIVEMA‡‡ + MTX††	20	+	6.8	Day 0	> 60	9/10
11	Day –7	DIVEMA-MTX**	20		6.8	Day 0	> 60	5/10
12	Day –7	DIVEMA-MTX††	20		6.8	Day 0	> 60	7/10
13	Day –7	MTX**	—		6.8	Day 0	13	0/10
14	Day –7	MTX††	—		6.8	Day 0	13	0/10

* Indicated groups received 1×10^7 irradiated L1210 cells ip on Day –7.
† Animals received drugs at indicated doses ip on Day –7.
‡ Dose of DIVEMA-MTX is expressed as DIVEMA and MTX equivalents.
§ Animals scored for survival at 60 days after challenge with 1×10^4 live L1210 cells ip on Day 0; MST = median survival time; S/T = survivors/total at time of scoring.
¶ DIVEMA, NSC-46015.
** Vehicle: 20% $NaHCO_3$, pH 8.3.
†† Vehicle: H_2O/NaOH, pH 7.3.
‡‡ DIVEMA, lot No. X20706-45, Hercules Inc.

immune-suppressive agent, MTX, would have an adverse effect on the adjuvant action of DIVEMA.

The results of two sets of experiments with either DIVEMA, DIVEMA-MTX, or the combination of DIVEMA + MTX are shown in table 2. Doses of 20 mg/kg of DIVEMA or DIVEMA equivalents were given ip. Animals receiving the vaccine only survived for > 90 days free of tumor, indicating complete inactivity of the L1210 vaccine. All control animals inoculated with live L1210 cells died from the leukemia with a median survival time of 9–10 days. Treatment with either 1×10^7 inactivated L1210 cells or DIVEMA alone produced only a small increase in survival of 2–3 days in mice challenged with tumor. However, 20 mg/kg of DIVEMA given in combination with the vaccine resulted in almost total resistance to tumor challenge, with 80%–100% 60-day survivors (treatment groups 5–8, table 2). No reduction of the protecting effect of DIVEMA was found when it was administered together with MTX (90% survivors). DIVEMA-MTX, at a 20-mg/kg DIVEMA equivalent dose, was still potent as an immunoadjuvant, but its effect was somewhat reduced compared to DIVEMA, with 50% and 70% survivors refractory to tumor challenge.

As expected, pretreatment with MTX alone had no effect on mice challenged with tumor cells 7 days later. Since previous experiments (10,11) had suggested that DIVEMA-MTX was labile to spontaneous hydrolysis of MTX from the polymer backbone, the potential effect of two different formulations, pH 8.3 ($NaHCO_3$) and 7.5 (titrated with NaOH), was studied (see table 2). However, there was no appreciable difference in the immune-potentiating effect on either DIVEMA or DIVEMA-MTX. Similarly, no difference in potency between the two different molecular weight specimens of DIVEMA used in this study could be observed.

Activation of Macrophages by DIVEMA and DIVEMA-MTX

The direct activation of macrophages by systemic treatment with DIVEMA has been clearly delineated as the mechanism responsible for DIVEMA's tumor growth inhibitor effect (15). Particularly, the highly immunogenic M109 lung carcinoma when used as a target cell in vitro was sensitive to DIVEMA (16). In contrast, this tumor in vivo is refractory to most chemotherapeutic agents (17). The well-characterized inhibition of DNA synthesis in M109 cells after exposure to DIVEMA-stimulated macrophages (16) appeared to be an appropriate test to study the comparative ability to DIVEMA-MTX to produce growth-inhibitory macrophages.

The cytostatic effects of mouse peritoneal macrophages harvested 6 days after ip treatment with two different DIVEMA equivalent doses are com-

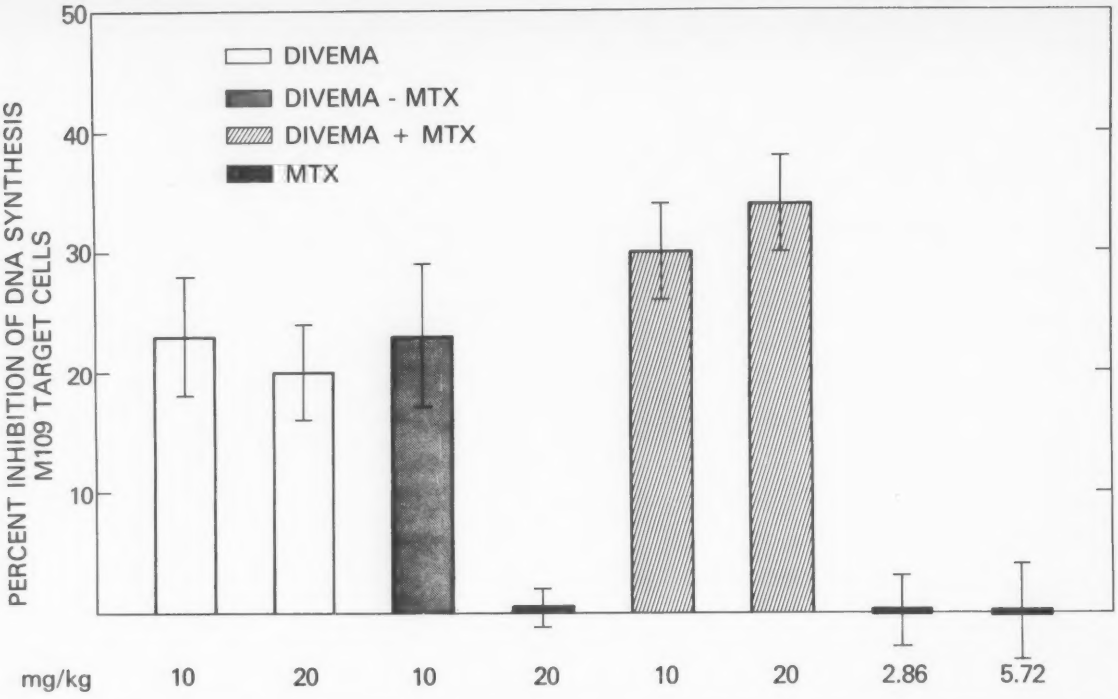

FIGURE 2.—Effect of DIVEMA and DIVEMA-MTX on the cytostatic activity of BALB/c peritoneal macrophages. Drugs at the indicated doses were given ip on Day 0. Macrophages were harvested 6 days later and tested for their ability to inhibit M109 cell DNA synthesis. The values represent the means ± SE of 2 experiments with triplicate samples. Doses of DIVEMA-MTX are expressed as DIVEMA equivalents; MTX doses are the actual MTX contents in the 10- and 20-mg/kg equivalent doses of DIVEMA-MTX.

pared in figure 2. DNA synthesis inhibition in M109 cells was similar after 10 or 20 mg/kg of DIVEMA, which represents approximately the optimal dose range to activate macrophages against this tumor (16). The combination of DIVEMA + MTX produced a slightly but insignificantly higher inhibitory effect of approximately 30% below the control level. Doses of MTX alone that corresponded to the MTX content in the DIVEMA-MTX copolymer were ineffective in activating macrophages. In contrast, the effect of DIVEMA-MTX was different at the two dose levels. A dose of 10 mg/kg of DIVEMA equivalents was similar in activity to DIVEMA. However, after 20 mg/kg, macrophages had completely lost their ability to inhibit DNA synthesis, whereas macrophage activation of DIVEMA alone has been reported to be active over a relatively broad dose range (18).

DISCUSSION

The functional role of macrophages activated by DIVEMA has been established in several experimental systems as a highly effective mechanism for providing immunopotentiation and antitumor activity. It was therefore of importance to determine this property of DIVEMA relative to other possible effects of DIVEMA-antitumor drug complexes. The results obtained with a DIVEMA-MTX copolymer presented here, in fact, indicate that the relative immune-potentiating action of DIVEMA is maintained, at least partially, both in a systemic intermittent and in an adjuvant treatment mode. DIVEMA-MTX as well as DIVEMA alone were active as adjuvants to increase the lifespan of L1210 leukemia-bearing mice treated with MTX. Therefore, this effect must be considered as one factor to explain the enhanced antitumor activity and long-term survival found in the treatment of murine L1210 leukemia with DIVEMA-MTX compared to MTX (11). It is, however, not possible at the present time to differentiate clearly the adjuvant effect of DIVEMA-MTX from the previously observed sustained plasma pharmacokinetic effect of MTX attained by fixation to this polymer in terms of their relative importance in the antitumor action of DIVEMA-MTX.

The activity of DIVEMA-MTX against Lewis lung carcinoma in mice (11) has led us to suggest a possible transport role of the activated macrophage for the copolymer. The uptake of DIVEMA by macrophages has been shown previously (19), using a ^{14}C-labeled copolymer. In the present study, we show that DIVEMA-MTX is capable of rendering macrophages cytostatic towards a solid murine tumor that is totally resistant to MTX, but its effect was diminished at a dose level (20 mg/kg) that maintained full potency for DIVEMA alone. It might be possible that, at this dose, the release of MTX from the polymeric carrier leads to a toxic effect towards macrophages, preventing their activation and cytostatic action against M109 carcinoma cells. Since a sharp dose dependence has been found for the macrophage-activating capabilities of several immunoadjuvants including DIVEMA (16), detailed studies at various dose ranges are required to characterize the effect of DIVEMA-MTX.

Little is known as yet about the relation of DIVEMA's chemical (polyanionic) structure to its ability to activate macrophages. The slightly reduced but still potent effect of DIVEMA-MTX to potentiate L1210 tumor cell vaccine would suggest that a *partial* substitution (neutralization) of the maleic acid groups still maintains the biologic activity of DIVEMA. The DIVEMA-MTX copolymer used in this study contained 25% MTX, but this polymer was previously found to be labile to spontaneous hydrolysis of MTX from the polymer backbone. The exact time course of the release of MTX has not yet been determined and is at present under study. Recently we have synthesized derivatives of DIVEMA substituted to different degrees with hydrophobic groups at the maleic acid moieties. The comparative investigation of these model compounds should provide a better understanding of the structural requirements of DIVEMA derivatives with regard to immune-potentiating ability, cytostatic action, and systemic toxicity.

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Cell and Tissue Distribution of ^{14}C-Labeled Pyran Copolymer

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SUMMARY

The tissue distribution of pyran (maleic anhydride-divinyl ether) copolymer was studied after a single ip injection of ^{14}C-labeled pyran (25 mg/kg) to mice. The pyran showed a reticuloendothelial distribution with the liver and spleen containing the highest concentrations which persisted for at least 21 days after drug treatment. Blood levels of ^{14}C-pyran reached a peak 2 hours after injection and were cleared within 6 hours. Attempts to measure uptake of ^{14}C-pyran by peritoneal macrophages were unsuccessful due to an inability to recover macrophages between 3 and 24 hours after ip pyran administration. Since activated macrophages appear to be the primary mechanism by which pyran enhances host resistance to microbial infection and neoplasia, the uptake of ^{14}C-pyran by isolated peritoneal macrophages in vitro was studied. Purified macrophages showed a gradually increasing uptake of ^{14}C-pyran, and a large amount of cell-associated radioactivity was bound to trichloroacetic acid-precipitable material. Several polyanions, including unlabeled pyran, dextran sulfate, and poly(I)·poly(C), competed for acid-precipitable receptor molecules. The superior antitumor effects of pyran as compared to other polyanions may result from the continuous presence of the synthetic polymer in the host. Possible mechanisms of immunopotentiation by pyran are discussed.

[Cancer Treat Rep 62:1845–1851, 1978]

Pyran copolymer, the polyanionic copolymer of maleic anhydride-divinyl ether, is a well-known interferon inducer (1) with a wide array of biologic activities. Aside from its anticoagulant and anti-inflammatory properties (2), it has received considerable attention because of its ability to enhance host resistance against various microbial infections (3,4) and neoplasms (5–7) and its ability to act as an adjuvant to viral (8) or radiation-inactivated (9) tumor cell vaccines. The protective effects of pyran appear to be largely due to an extensive stimulation of the reticuloendothelial system since nonspecifically activated macrophages can be recovered after systemic pyran therapy (10–12). Pyran treatment also produces a biphasic phagocytic response and enhances antibody formation in mice (13).

Pyran has been reported to be an effective adjuvant when used in concert with cytoreductive chemotherapy in two separate tumor systems. In a leukemia model system, pyran, when used as an adjuvant to chemotherapy, resulted in a significantly higher number of cures than obtained with chemotherapy alone (14). Similarly, in the Lewis lung carcinoma tumor model, pyran was also shown to be an effective adjuvant resulting in a significant increase in survival time compared to that achieved with chemotherapy alone. The potential use of pyran as an adjuvant in a clinical setting provoked the present study which was undertaken to assess the tissue distribution of pyran and to establish such parameters as tissue levels and pyran's interactions with cellular components representative of the immune system.

MATERIALS AND METHODS

Mice.—Male (BALB/c × DBA/2)F_1 (hereafter called $CD2F_1$) mice, 6–8 weeks old, were obtained from the Mammalian Genetics and Animal Production Section, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md). The animals were housed in

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plastic cages and fed Purina laboratory chow and tap water ad libitum.

Cells.—Adherent peritoneal macrophages were collected and purified as previously described (11). In addition, single cell suspensions were prepared from both spleen and thymus. The organs were excised aseptically and finely minced with scissors. Cell clumps were removed by filtering through sterile gauze, and rbcs were lysed by a 0.83% NH_4Cl solution. The macrophage-like J774 tumor cell line (15) was kindly provided by Dr. Peter Ralph, Sloan-Kettering Institute for Cancer Research (Rye, NY). An established line of lymphoblastic leukemia cells (MBL-2) was maintained in suspension culture. Cells were cultivated in RPMI-1640 medium supplemented with 20% fetal calf serum, 0.075% $NaHCO_3$, 10 mM Hepes buffer, and 100 μg/ml of gentamicin (RPMI-FCS).

Chemicals.—Pyran copolymer (NSC-46015) and ^{14}C-pyran copolymer (lot No. X20439-73) with a specific activity of 0.74 microcurie/mg were obtained from Dr. David Breslow of Hercules Research Center (Wilmington, Del). Pyran was synthesized with ^{14}C on either of two positions on the maleic anhydride polymeric backbone. The sodium salt of dextran sulfate (molecular weight, ~ 5×10^5) was purchased from Pharmacia Fine Chemicals (Piscataway, NJ) and poly(I)·poly(C) was supplied by Sigma Chemical Co (St. Louis, Mo). Chemicals were dissolved in Ca^{++} and Mg^{++} free Dulbecco's phosphate-buffered saline (PBS) and adjusted to pH 7.2 by the addition of 1 N NaOH.

Liquid Scintillation Counting

Tissue samples (100–200 mg) were minced with scissors and solubilized in 1 ml of Protosol (New England Nuclear Corp, Boston, Mass) until complete solubilization occurred. They were then cooled to room temperature, and 0.1 ml of 30% H_2O_2 was added. The samples were then reincubated at 55°C for 30 minutes, cooled down to room temperature, and 10 ml of Aquasol (New England Nuclear Corp) was added. The pH was adjusted to slightly acidic by addition of 1 N acetic acid.

Whole blood samples (100 μl) were solubilized in 0.5 ml of a mixture of Protosol:ethanol (1:2) for 1 hour at 55°C in a shaking water bath; they were then allowed to cool to room temperature, and 0.5 ml of a 30% H_2O_2 solution was added to each tube. The samples were reincubated for 30 minutes at 55°C, cooled to room temperature, and 15 ml of Aquasol was added. The pH was adjusted to slightly acidic by addition of 0.5 N HCl.

Samples consisting of washed cells were counted in Aquasol following three cycles of freeze-thawing. Trichloroacetic acid (TCA)-insoluble radioactivity was measured by trapping on glass-fiber paper (Millipore Corp, Bedford, Mass). Efficiency was calculated by the internal standard method using ^{14}C-glucosamine. The radioactivity of the samples was measured in a Packard Tri-Carb spectrometer.

RESULTS

Tissue distribution of pyran.—^{14}C-labeled pyran copolymer was given at a dose of 25 mg/kg by a single ip injection to mice, and tissue samples (100–200 mg) were processed for radioactivity up to 21 days after drug treatment. The pyran showed a reticuloendothelial distribution with liver and spleen containing the highest concentration which persisted for the duration of the experiment (fig 1). Blood levels of ^{14}C-labeled pyran reached a peak 2 hours after injection (1400 cpm/100 μl of blood) and were completely cleared within 6 hours. Significant amounts of pyran also remained in the intestine, kidney, and liver (fig 1). No activity was found in the brain at any of the time intervals tested.

Pyran uptake in vivo by peritoneal cells.—$CD2F_1$ mice, previously injected ip with 50 mg/kg of ^{14}C-pyran, were killed, and peritoneal cells were harvested at varying time intervals after pyran treatment. The peritoneal cells were washed three times in PBS and adjusted to 5×10^5 cells/ml. One-milliliter aliquots were freeze-thawed and the radioactivity in Aquasol was measured. A peak of uptake was observed at 2 hours after injection and was followed by a rapid decline (fig 2). In an attempt to measure uptake by macrophages and nonadherent cells following ip ^{14}C-pyran administration, peritoneal cells were harvested and resuspended at 4×10^6 leukocytes in 4 ml of RPMI-FCS. The cells were plated in 60-mm tissue culture dishes and allowed to incubate for 1 hour at 37°C in a 5% CO_2-in-air incubator. The nonadherent cells were removed by five cycles of aspiration with warmed (37°C) PBS. The adherent cells were resuspended in RPMI-FCS by scraping with a soft rubber policeman, and both adherent and nonadherent populations were quantitated with a hemocytometer. The adherent cells consisted of > 95% macrophages as judged by phagocytosis of latex beads and morphologic appearance. Immediately after injecting the pyran ip, the macrophages could not be recovered from the peritoneum (fig 3). Attempts to quantitate uptake of ^{14}C-pyran by macrophages were unsuccessful due to the inability to recover macrophages between 3 and 24 hours after pyran administration. After 3 days, macrophages again reached normal percentage levels.

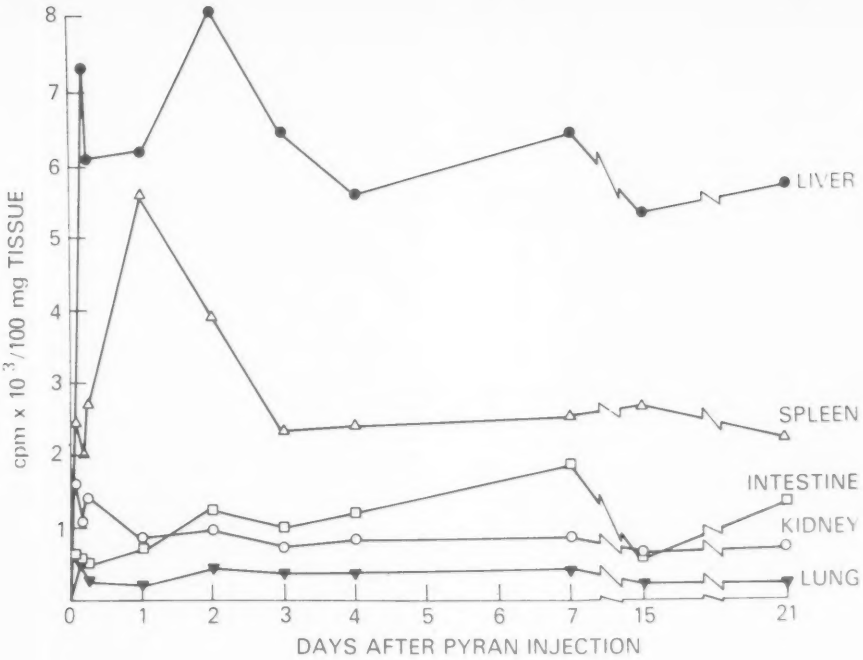

FIGURE 1.—Tissue distribution of ^{14}C-labeled pyran. CD2F$_1$ mice were injected ip with 25 mg/kg of ^{14}C-pyran. Tissue samples were taken at different time intervals after pyran administration and processed for measurement of radioactivity. Each point represents the mean of duplicate samples.

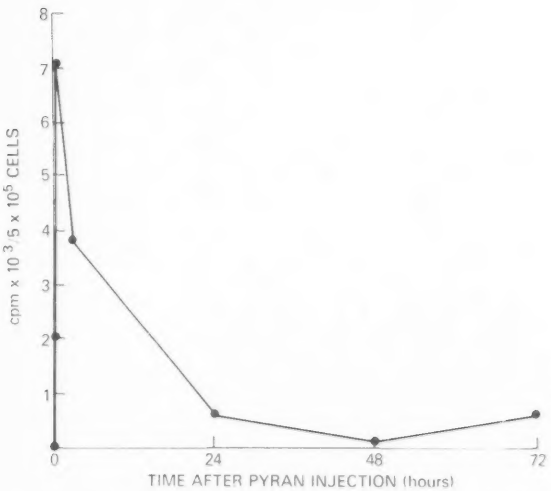

FIGURE 2.—Uptake of ^{14}C-pyran by peritoneal cells. 50 mg/kg of ^{14}C-labeled pyran was administered ip to CD2F$_1$ mice. Peritoneal cells were harvested at different time intervals, washed, and measured for radioactivity.

Pyran uptake in vitro by isolated macrophages, nonadherent peritoneal cells, and splenic lymphocytes.—Peritoneal macrophages, nonadherent peritoneal cells, and nonadherent spleen cells were exposed in vitro to ^{14}C-pyran at 20 μg/ml of culture medium. Peritoneal macrophages showed a gradually increasing uptake which reached a plateau after 9 hours (fig 4). In contrast, nonadherent peritoneal and spleen cells showed an initial rapid increase in ^{14}C-pyran uptake; this was followed by a fast decrease phase in the peritoneal cells, but not by the splenic cells, where uptake was maintained at moderate levels after the first hour of exposure.

Pyran uptake in vitro by the J774 macrophage-like cell line.—Exponentially growing J774 cells were exposed to ^{14}C-pyran in vitro, and cell-associated radioactivity was measured 3, 6, and 24 hours later. Cells were recovered from the cultures by shaking and washing three times with PBS prior to counting. Free ^{14}C-pyran was not precipitated by 10% TCA. However, a large amount of the cell-associated radioactivity (40%–87%) was bound to TCA-precipitable material at each of the time intervals tested (table 1). In another experiment, the neoplastic J774

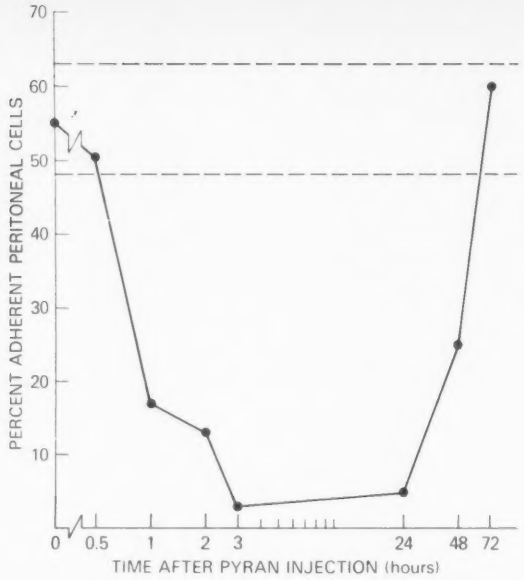

FIGURE 3.—Adherence of peritoneal cells after ip pyran (50 mg/kg) treatment. Peritoneal cells at 4×10^6 cells/4 ml of RPMI-FCS were plated in 60-mm tissue culture dishes and incubated at 37°C for 1 hr. Adherent cells were quantitated following removal of nonadherent cells by 5 cycles of aspiration with PBS. Horizontal dashed lines represent the normal % range of adherent cells in the resident peritoneal population.

cells were exposed to increasing concentrations of ^{14}C-pyran for 1 hour and TCA-insoluble radioactivity was measured (fig 5). The cell-associated pyran showed a saturation level at concentrations > 120 μg/ml. Under identical conditions, the TCA-insoluble radioactivity in normal macrophages was very low in comparison to the J774 cells.

Competition for pyran binding.—Several polyanions, including unlabeled pyran copolymer (NSC-46015), dextran sulfate, and poly(I)·poly(C), were tested for their ability to compete for binding of ^{14}C-pyran in J774 cells. Cells (1×10^6) in 1 ml of RPMI-FCS were simultaneously exposed to 10 μg/ml of ^{14}C-pyran and increasing concentrations of the other agents for 1 hour at 37°C. The cells were then washed three times with PBS, and TCA-insoluble radioactivity was measured. All polyanions competed for binding of ^{14}C-pyran to TCA-precipitable receptor molecules (fig 6).

DISCUSSION

Much of the antitumor and antimicrobial activity of pyran appears to be mediated by the nonspecifically activated macrophage. Once activated, the macrophage has increased ability to inhibit the growth of intracellular pathogens and selectively

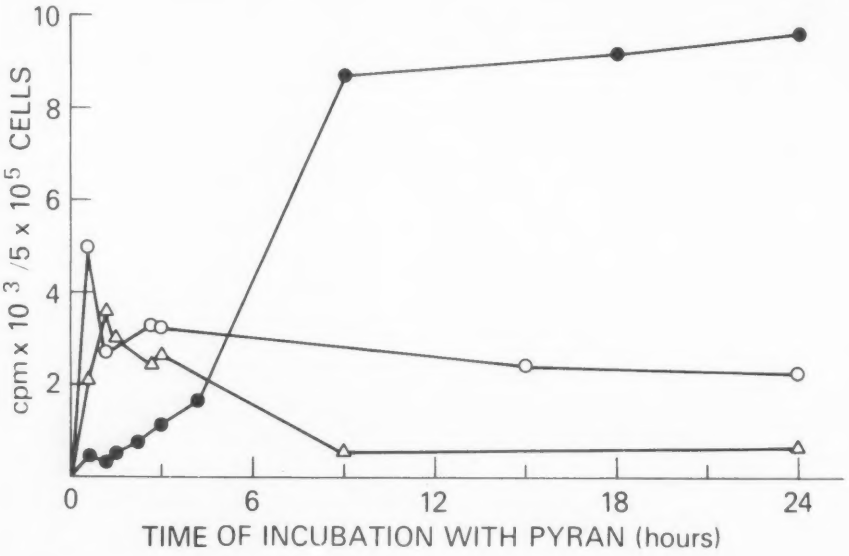

FIGURE 4.—Uptake of ^{14}C-pyran by peritoneal macrophages (●) and nonadherent peritoneal (△) and spleen (○) cells. Isolated cells, depleted of rbcs, were suspended at 10^6 cells/4 ml of RPMI-FCS in 35-mm tissue culture dishes. Each culture was treated with 20 μg/ml of ^{14}C-labeled pyran and incubated at 37°C. After different time intervals, cells were removed from triplicate cultures and washed with PBS, and the radioactivity of freeze-thawed samples was determined.

TABLE 1.—Cell-associated (CA) or TCA-precipitable radioactivity in J774 cells exposed to ^{14}C-pyran in vitro*

Pyran (μg/ml)	Time of incubation with pyran					
	3 hrs		6 hrs		24 hrs	
	CA†	TCA†	CA	TCA	CA	TCA
10	3593	2512	2841	2053	13,750	6258
20	4329	3745	6223	4918	38,532	15,500

*Triplicate cultures containing 10^6 cells in 3 ml of RPMI-FCS were incubated in the presence of ^{14}C-pyran.

†Radioactivity is expressed as dpm/5 × 10^5 cells.

kills transformed cells by a cell-to-cell contact mechanism (16). We have previously shown that pyran's effect is directly on resting macrophages and probably not produced by lymphocytic mediators (11). Although the exact mechanism of macrophage stimulation by pyran is presently unknown, at least two distinct possibilities exist. Pyran may activate macrophages via interferon induction since macrophage cultures produce interferon after pyran treatment in vitro (17) and since fibroblast-derived interferon has been demonstrated to be a potent macrophage activator (18). On the other hand, pyran may behave as a model gene regulator, binding to nuclear histones and releasing template restriction (19).

In vivo and in vitro evidence demonstrates that pyran distributes itself and persists in organs rich in mononuclear phagocytes such as the liver and spleen. It apparently enters the cell by endocytosis and binds nonspecifically to intracellular receptors due to its polyanionic nature. Evidence supporting the role of the polyanionic nature of the molecule in receptor site reactivity was demonstrated when other polyanions such as poly(I)·poly(C) and dextran

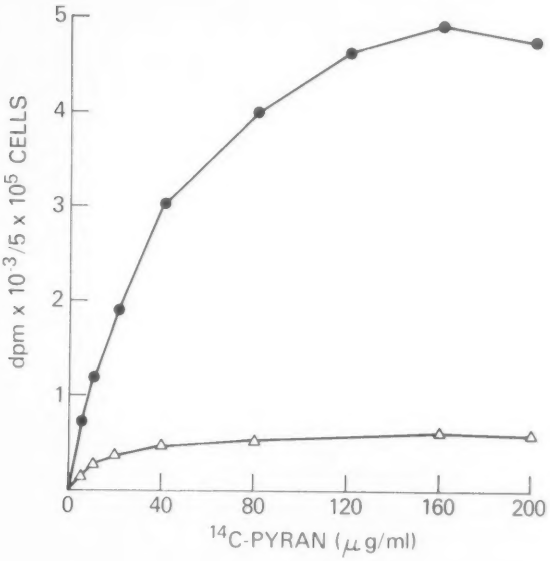

FIGURE 5.—Acid-precipitable radioactivity bound to J774 neoplastic macrophages (●) and normal peritoneal macrophages (△). Triplicate cultures containing 10^6 cells/2 ml of RPMI-FCS were treated with increasing concentrations of ^{14}C-pyran for 1 hr at 37°C. Following incubation, the cells were washed twice in PBS and TCA-precipitable radioactivity was measured.

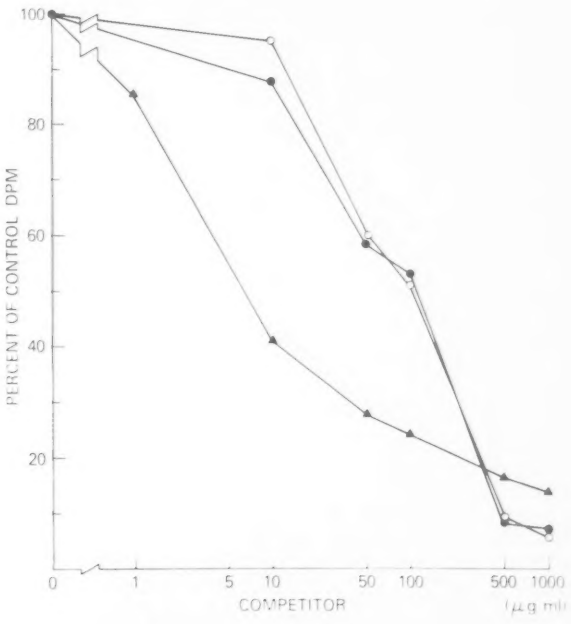

FIGURE 6.—Competition of ^{14}C-pyran binding by unlabeled pyran (●), dextran sulfate (▲), and poly(I)·poly(C) (○). Cultures containing 10^6 J774 cells in 2 ml of RPMI-FCS were exposed to 10 μg/ml of ^{14}C-pyran or mixtures of ^{14}C-pyran plus the competitor polyanion, and incubated for 2 hrs at 37°C. The recovered cells were enumerated and TCA-insoluble radioactivity was measured. Results are expressed as % of the uncompeted control dpm.

sulfate competed for binding with ^{14}C-pyran. In the present study it was of interest to note that within 3 hours after ip pyran treatment, peritoneal macrophages were not recoverable. After 3 days, the number of macrophages in the peritoneal flora again reached normal control levels. The timing coincides with the observations that macrophages recovered from the peritoneal cavity after pyran injection were cytotoxic to tumor cells (20). However, maximum cytotoxicity was not manifested by peritoneal macrophages until 6 days after pyran administration. Similar findings of inability to recover macrophages and time course of activation have been observed for two other polyanionic interferon inducers, endotoxin and poly(I)·poly(C) (21). Acid-insoluble radioactivity can be detected in purified nuclei early after exposure of intact macrophages to ^{14}C-pyran in vitro. Such pyran could form strong complexes with histones and result in destabilization of nucleoprotein and subsequent release of template inhibition (19,22).

Pyran has also been shown to enhance antibody formation in mice (13). It is possible that the ability of pyran to complex with Ca^{++} may control the differentiating step(s) leading to antibody-forming cells (23). Studies in our laboratory have shown that pyran is weakly mitogenic for spleen cells in vitro with peak stimulation at approximately 50 μg/ml (unpublished finding); however, the mitogenicity of pyran was considerably less than that of either concanavalin A or lipopolysaccharide. The kinetics of pyran uptake in vitro by splenic lymphocytes was quite different from that of macrophages. Lymphocytes showed an initial rapid increase in ^{14}C-pyran uptake which peaked within 1 hour and was followed by a gradual decline. In contrast, macrophage uptake was relatively slow but progressive, reaching a maximum by 9 hours and maintained at this peak for up to 24 hours. These results would indicate that pyran is, indeed, retained by the macrophage.

In conclusion, pyran is concentrated and remains for a long period of time in reticuloendothelial organs such as the spleen and liver. Although it is not known whether the radioactivity measured still reflects active pyran molecules, pyran's superiority as an immunopotentiator over other polyanions may be related to a longer biologic half-life. Goren et al have shown that certain polyanions prevent phagosome-lysosome fusion in cultured macrophages, thus avoiding exposure to the lysosomal hydrolases (24). Whether this is the case with pyran remains to be determined. The ability of pyran copolymer to act as an immunopotentiator and enhance antitumor resistance is presently under intensive investigation.

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Future Direction of Synthetic Polyanions (Pyran Copolymer)

William Regelson [1,*]

SUMMARY

Pyran copolymer (maleic anhydride-divinyl ether) has consistently reproducible molecular weight-related biologic effects associated with toxic, immunologic, antitumor, and antiviral effects. Fortunately, the antitumor action occurs with the least toxic lower molecular weight fraction. Immunoadjuvant effects with this fraction would be critical to its development. Studies of polymers should include evaluation of effects on splenomegaly, splenic esterase changes, lipolysis, reverse transcriptase, nucleases, calcium flux, cyclic nucleotides, and complement and clotting elements.

[Cancer Treat Rep 62:1853–1856, 1978]

Pyran copolymer, or maleic anhydride-divinyl ether (MVE) copolymers, represents some 12–13 years of effort to develop antitumor and/or antiviral agents which produce a wide range of biologic activity through effects on host modulation (1–5). The experience with pyran was antedated by Monsanto-derived MVE copolymers (6,7), and this stemmed from earlier work with synthetic polysulfonates and polyphosphates which extended from studies with heparin or naturally derived heparinoids (3,6–10). In many ways, these polyanions resemble the synthetic polynucleotides, and their biologic action is once more emphasized by the strong electronegative charge on thymosin as has been reported by A. Goldstein in a report presented at this meeting.

What has been presented in regard to MVE fractions represents a reaffirmation of what was previously reported for pyran, namely, that the higher the molecular weight, the more toxic in relation to interference with nonspecific drug-metabolizing enzymes and the production of anemia, leukocytosis, thrombocytopenia, and endotoxin sensitization (1–3,5,10–12). These findings, which are distinct from the anticoagulant effect of pyran, will culminate in the rediscovery of the clinical toxicity (fever, thrombocytopenia, hypertension, and possibly transient blindness and/or hemolysis [1]) unless strict attention is paid to the clinical development of an MVE polymer with a molecular weight of $< 15{,}000$.

A molecular weight of 15,000 is not only the apparent toxicity cut-off, but it reaffirms the observations for pyran that the antitumor activity is preserved while the antiviral and perhaps immunostimulatory activity is decreased with the MVE fraction in the 12,000-molecular weight range. Investigation of dose-related or cumulative toxicity and biologic effects for the 12,000-molecular weight fraction, as well as its immunoadjuvant and anticoagulant activity, must be performed.

The following is relevant to recent observations and/or previously reported experience that was not discussed at this meeting.

Splenomegaly

Regardless of molecular weight, splenomegaly is seen in mice receiving pyran in perhaps a dose-related relationship for the lower molecular weight material as reported by Morahan et al at this meeting. This is important because of recent observations by Kaplan et al (12) that *Corynebacterium parvum* derived from different *Corynebacterium* strains shows antitumor activity only when splenomegaly is present. If, as in the case of *C. parvum*'s antitumor activity, splenomegaly is a clue to pyran's antitumor activity, then it is obvious that we must look to what is occurring in the spleen to provide

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insight into the mechanism of action for pyran copolymer. In this regard, morphologic observations show the loss of splenic germinal centers with macrophage mobilization following pyran administration (13).

What are the reticuloendothelial cells mobilized by pyran? Does the new 12,000-molecular weight pyran fraction (MVE) produce cytoplasmic inclusions? What are these cytoplasmic inclusions, how are they derived, and is their presence a clue to the antitumor or immunoadjuvant activity seen? The significance of polyanion-induced cytoplasmic inclusions has been discussed in several reviews (1–3) since they are also induced by tilorone and related congeners and compounds such as atabrine which have reverse transcriptase-inhibiting activity.

Esterase

In regard to the above, we must look at the observations by Roberts and Regelson (14) that pyran-induced BAME and TAME splenic esterase (fibrinolytic) activity was associated with the resistance of mice to Friend leukemia virus. This depended on the route of pyran administration, since iv pyran decreased splenic esterase while ip pyran increased it, with Friend leukemia virus susceptibilities varying similarly (15).

The pertinence of these observations regarding esterase changes may be appropriate to inhibition or activation of complement or changes of metabolism that should be explored.

Anticoagulant-Lipolytic Activity

The original work with MVE synthetic polyanions was related to the experience of I. Page, who in an attempt to develop lipemia-clearing agents with minimal anticoagulant activity, studied these compounds for Monsanto (6,7). Synthetic polyanions behave like heparin and can activate lipases and/or lipase co-factors or possess physical-chemical characteristics that produce lipemia clearing through colloidal dispersion. This is particularly pertinent in view of the recent observations which indicate that low-density lipoproteins inhibit lymphoblastic transformation and may act as immunosuppressors (16).

In addition, Kos et al (17) have recently shown that chronic coxsackievirus infection, in association with high lipid feeding, results in hypercholesterolemia with suppression of the immune response and the concomitant growth of tumors in these immunosuppressed mice. The activity of these synthetic polyanions could conceivably relate to their capacity to alter lipoproteins and/or cholesterol distribution.

The clinical pyran (NSC-46015) with a molecular weight averaging 18,000–23,000 is an excellent anticoagulant which blocks the polymerization of fibrinogen to fibrin (18,19). In clinical trials, no in vivo hemorrhage was seen and pyran's action as an anticoagulant is species-related (1). Pyran is an excellent in vitro anticoagulant for clinical laboratory action as a substitute for heparin. It remains to be seen if the lower molecular weight fractions (12,000) which are less toxic have in vivo clinical value.

Anticomplement or Complement-Enhancing Activity

Synthetic polyanions including pyran copolymer can inhibit the various complement fractions and/or activate the C3 bypass (1,2). Recent work has demonstrated that fatty acid incorporation in target cells can interfere with complement-induced cell lysis (20). Exploration of the possibility that synthetic polyanions or activated macrophages can interfere with the mobilization and cellular absorption of free fatty acids might explain how pyran could make tumor or virus-infected cells lethally susceptible to the action of macrophages or serum complement.

Stimulation of Drug Hydroxylases

One of the major toxicologic side effects of increasing molecular weight of pyran copolymer has been the inhibition of nonspecific P450 drug hydroxylases of the liver (21). The recent observations that these hydroxylases are also present in the nucleus and can be stimulated or blocked by the same factors which do this in the endoplasmic reticulum of the liver (22) suggest that the action of pyran copolymer on the nucleus and nuclear drug-metabolizing enzymes could have some relation on the effect of these agents on tumor growth and/or viruses. It should be remembered that nonspecific hydrolases can produce free radicals which can cause tissue injury, which may be one explanation for their mechanism of action.

Reverse Transcriptase and Nuclease Inhibition

In the search for mechanisms of action, it should be remembered that pyran copolymer and other synthetic polyanions can inhibit reverse transcriptase (1–3,23). The inhibition of nucleases, both ribonucleases and deoxyribonucleases, is also seen for polyanions (3,6,7). Polyanions have, as have polycations, been used to stabilize RNA-protecting RNA from RNAse digestion for immunologic transfer

purposes. Certainly, a search for molecular weight differences for the MVE (pyran) fractions is something that we should consider with particular reference to antitumor and/or antiviral immunoadjuvant action.

Immune Stimulation

In regard to host immune response, it should be remembered that thymosin, by protein electrophoresis, is one of the most anionic peptides, and perhaps the anionic character of thymosin may indicate how the synthetic polyanions function. Thus, it may be that the synthetic polyanions, because of their negative charge effect, can reduplicate the action of thymosin.

Vaccine: Immunoadjuvant Action (Antitumor and Antivirus)

We must re-emphasize the experience of Chirigos' group in regard to the immunoadjuvant action of pyran in stimulating host resistance to irradiated tumor cells and tumor viruses (24). Similarly, we must apply the observations of Campbell and Richmond (25) wherein pyran acts as an extremely potent adjuvant to turn an ineffective antiviral vaccine into an effective one. We should look to see if tumor-specific antigens such as carcinoembryonic antigen, alpha-fetoprotein, tumor parahormones, and/or glycoproteins, which characterize tumor cell antigenicity, could be used in conjunction with synthetic polyanions to make more specific antitumor vaccines.

Anti-Inflammatory and Adjuvant-Induced Arthritis Blocking Action

Polyanions have a clinical history of anti-inflammatory effects (3,6–7). In this regard, polyphloretinine phosphate and polyethylene sulfonate have been used topically (1) and pyran can prevent adjuvant-induced arthritis in rats. Will the low molecular weight MVE fraction (12,000) behave similarly? If so, it may be a useful topical or systemic agent in allergy or hyperimmune disease.

Macrophage Compartmentalization

In another area, we must remember that macrophages are compartmentalized within tissue or regional areas. In addition, MVE copolymers can stimulate granulocytosis (1–3) and granulocytes can block the colony-stimulating activity of macrophages while activated macrophages can block lymphoblastic transformation, and heparin, the native polyanion, stimulates lymphocytosis. We need to use polyanions as trophic agents to control wbc interrelationships.

In making synthetic adjuvants or other immunoadjuvants effective we must recognize that there is a localization of macrophage effect based upon their ability to enter into the area where their activity is desired. Peritoneal exudate cells have difficulty getting into the general circulation and we must explore techniques to stimulate killer macrophages to enter into the tumor for selective inhibition. The coupling of synthetic polyanions to leukotaxins or leukotoxins could be an interesting way to introduce them into tissue areas where their activity is needed.

Calcium Flux

Finally, it must be stressed that preliminary work of R. Carchman (personal communication, 1977) indicates that macrophage activation is associated with a shift of calcium, possibly from mitochondria within the cell. The effect of calcium ionophores, from the point of view of morphologic alteration of the tumor target cell, may bear some physical resemblance to what is seen when polyanions or activated macrophages inter-react with the tumor cell surface. What is common in regard to the morphologic events associated with macrophages or polyanion-induced cell death, at least superficially, resembles what one sees with that produced by calcium ionophores.

Recent work that we have done in collaboration with DeBrobander and Brogers from Janssen Laboratories (work in progress, 1978) suggests that pyran copolymer may increase the level of microtubulin within the cell. That this may be true is emphasized by preliminary work regarding cyclic GMP elevation in activated macrophages (Kaplan, Carchman, and Regelson, work in progress, 1978).

Enzyme Inhibition

It must be remembered that synthetic polyanions are "anti-inflammatory agents" and, in addition, inhibit enzymes such as acid phosphatase and alkaline phosphatase (6,7). It should also be remembered that levamisole has the capacity to inhibit alkaline phosphatase which is something that should be seen as a possible common step between both agents which stimulate host resistance.

In conclusion, I urge that all of us working with synthetic polyanions should not forget what has been done by earlier investigators. We must remember that polyanions alter the isoelectric point of protein; this goes back to the original work of A.

Fisher (6,7) who felt that this was the mechanism by which it controlled growth through interfering with the passage of protein or polypeptides within the cell. Heilbrun felt that changes in calcium flux ("calcium precipitation") by polyanions could alter sol-gel relationships and block cell division (6,7). In this regard, it should be remembered that polyanions can bind or decorporate metals and these observations are pertinent to the potential usefulness of pyran in ridding the liver of bound plutonium.

It has been most unfortunate that the approach to the study of synthetic polyanions has not had the coordinating resources that could conceivably have come from the chemical industry which gave rise to them. However, pharmaceutic interest is changing this, as can be seen in the recent IUPAC International Conference on Biopolymers which was presented in Prague in August 1977. From this and other indications, it can be seen that the place for the application of biopolymers in clinical medicine is just beginning.

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Glucan: Inhibition of Tumor Growth and Enhancement of Survival in Four Syngeneic Murine Tumor Models [1]

N. R. Di Luzio,* R. McNamee, W. I. Browder, and D. Williams [2]

SUMMARY

Previous studies have demonstrated that glucan, a β1,3-polyglucose derived from the cell wall of *Saccharomyces cerevisiae*, is a potent stimulant of the reticuloendothelial system as well as of cellular and humoral immune responses. In view of the demonstrated effectiveness of intralesional glucan administration in a variety of human neoplasia and in a rat acute myelogenous leukemic model, studies were initiated to evaluate the effectiveness of glucan in mediating antitumor activity in a variety of syngeneic murine tumor models. Murine malignancies selected for these studies included the BW10232 adenocarcinoma, anaplastic 15091A mammary carcinoma, B16 melanoma, and AKR lymphocytic leukemia. The administration of glucan iv after the sc administration of carcinoma or melanoma cells in respective host strains of mice was markedly effective in limiting neoplastic proliferation. The decreased tumor weight averaged approximately ≥ 70% in glucan-treated mice as compared to controls. Histologic examination of mice with melanoma or leukemia also demonstrated a reduction in metastases. Iv glucan administration prolonged survival of mice with either melanoma, adenocarcinoma, anaplastic carcinoma, or lymphocytic leukemia. However, in the adenocarcinoma model, ip administration of glucan was totally ineffective in modifying survival. Conjoint surgical resection and glucan administration was more efficacious in extending survival in mice with melanoma than either treatment alone. These studies further delineate a therapeutic potential for glucan.

[Cancer Treat Rep 62:1857–1866, 1978]

In recent years, attempts have been made to alter the course of neoplasia by augmenting host-defense mechanisms. A number of adjuvants including bacterial vaccines and chemical compounds have been demonstrated to produce a nonspecific stimulation of the reticuloendothelial system and augment humoral and cellular immune response. The most commonly known bacterial agents are bacillus Calmette-Guérin (BCG) and *Corynebacterium parvum* (1,2). However, the efficacy of such nonspecific immunotherapy varies, depending upon the time and mode of injection (3–5) as well as the type of malignancy (4,6). In view of the virulent nature of bacterial adjuvants such as BCG, it is not surprising that complications have been observed on occasion following intratumor or intradermal injections of these agents (2,7). Furthermore, there is the threat of disseminated infection following inoculation with viable organisms in patients with immunodeficient states secondary to malignancy (2). Indeed, Sparks et al (8) reported that rats receiving isonicotinic acid hydrazine and BCG intralesionally had better survival and smaller tumor volumes than rats receiving BCG alone, possibly by inhibiting diversion of host response to the viable BCG organism.

In general, the currently employed adjuvants do not meet many of the desirable criteria developed in this laboratory (9) and outlined in a recent World Health Organization Technical Report (Immunological Adjuvants: WHO Technical Report, Series No.

[1]Supported in part by the Cancer Research Institute of New York, MECO Cancer Research Fund, and the American Cancer Society, Inc.

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595). Aside from bacterial adjuvants, previous studies have demonstrated that glucan, a β1,3-glucosidic polyglucose derived from the cell wall of *Saccharomyces cerevisiae*, is a potent immunopotentiator (7,9). The administration of glucan to mice or rats is associated with the induction of a hyperphagocytic state and the hypertrophy of major reticuloendothelial organs due to an increase in both the size and number of macrophages (7,9,10). This neutral polyglucose is effective in enhancing both humoral and cellular immune responses to unrelated antigens (11,12). Additionally, glucan is nonantigenic (13) and nonvirulent as opposed to bacterial immunostimulants and may, therefore, be anticipated to induce fewer toxic complications. The potential of this polyglucose as an immunotherapeutic agent in the treatment of malignancy remains to be fully appreciated. Initial studies have demonstrated that the iv administration of glucan prior to administration of Shay acute myelogenous leukemia tumor cells resulted in significant modification of tumor growth and prolongation of survival (14). Glucan significantly modified tumor growth in this same allogeneic tumor model, in which the primary tumor approximated 2%–3% of body weight and metastases to internal organs had developed (14). In clinical studies involving a variety of metastatic lesions in man, intralesional administration of glucan produced a prompt necrosis of the tumors associated with a pronounced monocytic infiltrate (13,15). From these previous experiments, it is apparent that glucan can markedly modify host resistance to neoplasia.

In spite of the effectiveness of glucan upon intralesional administration in man, objections have been raised concerning the employment of the Shay acute myelogenous leukemia model because of its possible allogeneic nature (16). This model was specifically selected because it was found to completely reproduce the alterations in plasma opsonic activity which were noted in patients with diverse malignancies (17). The relatively nonimmunogeneic nature of this tumor was also demonstrated since attempts to immunize rats against Shay acute myelogenous cells were found to be unsuccessful unless glucan was employed as an adjuvant (18).

The purpose of this present study was to further evaluate glucan's ability to inhibit tumor growth in a variety of syngeneic tumor models. Four separate murine malignancies of spontaneous origin including the anaplastic 15091A mammary carcinoma, BW10232 adenocarcinoma, B16 melanoma, and AKR lymphocytic leukemia were selected for these studies. Both tumor weight and survival were monitored following systemic administration of glucan.

TABLE 1.—Origin and source of syngeneic mouse tumor models

Tumor	Origin	Host mouse strain
Adenocarcinoma (BW10232)	Spontaneous, mammary gland	C57BL/6J
Anaplastic mammary carcinoma (15091A)	Spontaneous, mammary gland	A/J
B16 melanoma	Spontaneous, skin at base of ear	C57BL/6J
Lymphocytic leukemia (BW5147)	Spontaneous, spleen and nodes	AKR/J

The extent of metastatic involvement in experimental and control groups was ascertained by light microscopic studies employing the melanoma tumor. Additional studies were conducted to evaluate the efficacy of glucan as an adjunct to surgical resection in prolonging survival of mice with sc implanted melanoma.

MATERIALS AND METHODS

Male C57BL/6J, AKR/J, and A/J inbred mice (Jackson Laboratory, Bar Harbor, Me) were employed as recipients for the respective syngeneic tumors listed in table 1. The mice were maintained on Purina laboratory chow and water ad libitum. All tumors were excised and dispersed under sterile conditions by means of a glass tissue grinder. The number of viable cells was determined by trypan blue exclusion. Established dilutions of the cells were then injected sc in the appropriate mouse strain. AKR lymphocytic leukemia cells for iv injection were obtained from culture. Tumor cells were maintained as suspension cultures in RPMI-1640 medium supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 units/ml). The cells were cultured in sterile plastic flasks (Falcon Plastics, Oxnard, Calif) at 37°C and 5% CO_2.

Yeast glucan was isolated from *S. cerevisiae* by a modification of the method of Hassid et al (19). The size of the particulate glucan prepared in this fashion is approximately 1 μm (13). Since the dose and schedule of treatment varied for many of the experimental procedures, details as to experimental design are incorporated in each experimental phase.

For light microscopic studies, organs were dissected from mice and 0.5-cm slices were fixed in 4% buffered formalin. Tissues were dehydrated in a graded series of ethanol, cleared in xylene, and embedded in paraffin. Sections, 6 μ thick, were stained with hematoxylin and eosin.

TABLE 2.—Inhibitory influence of glucan on growth of B16 melanoma*

Group	No. of mice	Weight (g) of—†		
		Liver	Spleen	Tumor
Control	29	1.21 ± 0.06	0.18 ± 0.01	0.97 ± 0.19
Glucan	23	2.17 ± 0.09‡	0.48 ± 0.41‡	0.26 ± 0.08‡

*1 × 10^6 tumor cells were injected on Day 0. Glucan (0.3 mg/mouse) was administered on Days 3, 6, 9, 12, and 14. Animals were killed on Day 16 for determinations of organ and tumor weights.

†Values are expressed as means ± standard error.

‡Significant ($P < 0.05$).

Data were analyzed for statistical significance by either the Student's t test or chi-square test. All data were compared for statistical significance employing a confidence level of 95%.

RESULTS

Effect of Glucan on Tumor Growth and Survival of C57BL/6J Mice With B16 Melanoma Implants

To evaluate the effect of iv administered glucan on primary tumor growth, mice were injected with 1 × 10^6 melanoma cells, and glucan (0.3 mg/mouse) or saline was administered on Days 3, 6, 9, 12, and 14 (table 2). When the animals were killed on Day 16, tumor weights were decreased by approximately 70% ($P < 0.05$) in the glucan-treated group. Twelve of the 23 glucan-treated mice had no detectable subcutaneous tumors in contrast to only one of 29 control animals. Significant hepatomegaly and splenomegaly were associated with the reduced tumor growth in glucan-treated mice (table 2).

Histologic examination of the liver and lung was also done on Day 16. None of the animals in either the glucan or control group exhibited metastatic lesions of the liver. Livers in the glucan-treated mice were characterized by the presence of granuloma and a hyperplasia of Kupffer cells. Analyses of lung histology revealed metastatic tumor in eight of nine control mice in which studies were undertaken. Tumor growth was prominent throughout the lung parenchyma. In marked contrast, none of the glucan animals had detectable tumor nodules in the lung. As with the liver, however, lung tissue in mice treated with glucan exhibited hyperplasia of macrophages and granuloma formation.

To further evaluate the efficacy of glucan as a posttreatment modality, the death rate was evaluated following an sc injection of 1 × 10^6 melanoma cells on Day 0 and subsequent iv injections of glucan (0.3 mg/mouse) at 3-day intervals over a 12-day period. Glucan was effective in prolonging survival as denoted by the marked disparity in the death rate (fig 1). The median survival time was 20 days in saline-treated control mice after tumor cell inoculation in contrast to 28 days in glucan-treated mice ($P < 0.05$). Glucan, therefore, significantly altered the course of melanoma as reflected by reduced tumor growth, metastases, and increased survival time.

It is apparent that if glucan is to be employed clinically as an immunotherapeutic agent, one of its greatest attributes would be the ability of glucan to reduce residual tumor size or disseminated lesions following surgical resection. Studies were initiated, therefore, in which B16 melanoma tumor cells (1 × 10^6 cells/mouse) were injected sc on Day 0. On Day 15, the subcutaneous tumor was surgically resected and the wound was sutured. Groups of mice received glucan iv (0.45 mg/mouse) or saline at the time of resection and on Days 5, 3, and 2 prior to surgery as well as on Days 2, 4, and 6 following surgery. Surgical resection alone in saline-injected control mice significantly prolonged survival relative to control mice that did not have resections (fig 2 vs fig 1). This enhanced survival time was denoted by the median survival of 33 days in mice in whom the melanoma was resected (fig 2) as compared to an equivalent survival time of 20 days in animals not receiving resections (fig 1). Of significance, however, was the difference in length of survival time between saline-control mice with resections and mice receiving preoperative and postoperative immunotherapy with glucan. In contrast to the 50% death rate within 33 days of the tumor-resected control mice, the median survival time in the glucan-treated tumor-resected group was extended to 43 days ($P < 0.01$). Glucan, therefore, proved to be an effective adjunct to surgical resection.

Antitumor Activity of Glucan in C57BL/6J Mice With BW10232 Adenocarcinoma

The adenocarcinoma (5 × 10^6 cells/mouse) was administered sc and followed by iv saline or glucan injections (0.2 mg/mouse) on Days 1, 6, 10, and 15

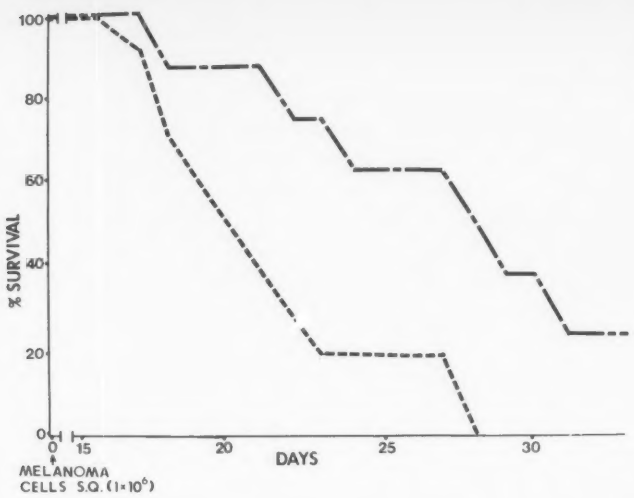

FIGURE 1.—Death rate of glucan- and saline-treated mice after sc injection of 1×10^6 melanoma cells. Glucan (——) was administered iv (0.3 mg/mouse) on Days 0, 3, 6, 9, and 12; total No. = 8. Saline control animals (- - - -) received isovolumetric saline; total No. = 10.

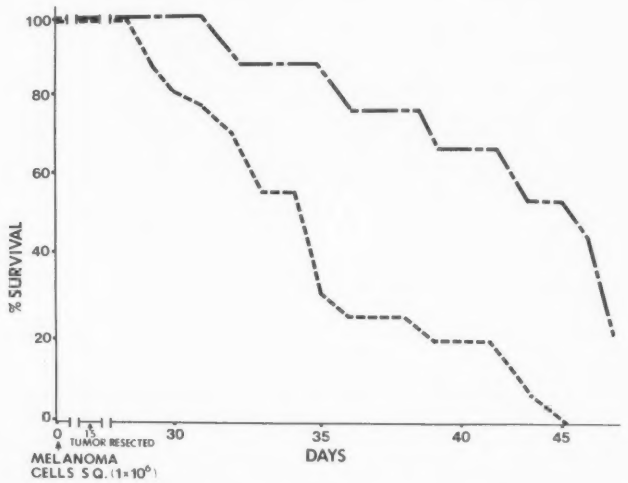

FIGURE 2.—Compatability of surgical resection with glucan immunotherapy. Tumors were surgically resected on Day 15 after sc challenge with 1×10^6 melanoma cells. Glucan-treated animals (——) were injected on Days 5, 3, and 2 prior to and on Days 2, 4, and 6 following surgery as well as on the day of resection. Saline controls (- - - -) received an equivalent regimen of isovolumetric saline; total No. = 9.

following the administration of tumor cells. The mean tumor weight on Day 21 was 4.21 g in the control groups as opposed to 0.99 g in glucan-treated mice (table 3). This inhibitory effect of glucan on neoplastic proliferation is represented by a 77% reduction in tumor weight. In accordance with previous experiments, there was significant hepatomegaly in the glucan-treated mice, denoting hypertrophy and hyperplasia of macrophage cells.

To assess if a higher dose of glucan would be even more efficacious in limiting growth of the tumor, the glucan dose was increased to 0.45 mg/mouse and administered iv at 2-day intervals after sc injection of the adenocarcinoma cells (table 3). The increased

TABLE 3.—Inhibitory effect of iv glucan on sc growth of adenocarcinoma (BW10232)

Group	Weight (g) of—		
	Liver	Spleen	Tumor
Experiment 1*			
Control	1.56 ± 0.11	0.35 ± 0.03	4.21 ± 0.08
Glucan	1.82 ± 0.09‡	0.97 ± 0.01‡	0.98 ± 0.15‡
Experiment 2†			
Control	1.33 ± 0.03	0.12 ± 0.01	0.59 ± 0.17
Glucan	1.84 ± 0.13‡	0.40 ± 0.02‡	0.09 ± 0.01‡

*C57BL/6J mice were injected with 5×10^6 cells/mouse. Glucan (0.2 mg/mouse) or saline was administered on Days 1, 6, 10, and 15 following tumor cell challenge. Tumors were excised on Day 21 (9 controls and 6 glucan-treated mice).

†C57BL/6J mice were injected sc with 1×10^6 adenocarcinoma cells. Glucan (0.45 mg/mouse) or saline was administered on Days 0, 2, 4, 6, 8, and 10. Tumors were excised on Day 12 (9 controls and 9 glucan-treated mice).

‡Significant ($P < 0.01$).

dose of glucan was extremely effective in retarding growth of the adenocarcinoma as denoted by an 85% decrease ($P < 0.01$) in tumor weights of glucan groups relative to the control group on Day 12 after tumor cell injection.

As with the melanoma tumor, studies were initiated to evaluate the effect of glucan on survival time of the mice following the sc administration of mammary adenocarcinoma cells. Injection of glucan at 3-day intervals at a dose of 0.45 mg/mouse resulted in prolonged survival. This protective effect is denoted by the 100% survival of glucan-treated mice at Day 19 compared to only 20% survival of control mice (fig 3). However, the cessation of glucan administration on Day 18 appeared to be associated with rapid onset of death.

In contrast to the protective effect of iv administered glucan in prolonging mean survival time, the ip injection of glucan (1 mg/mouse) on Days 1, 6, 10, and 15 after sc administration of 5×10^5 adenocarcinoma cells was not effective in modifying mortality patterns (fig 4).

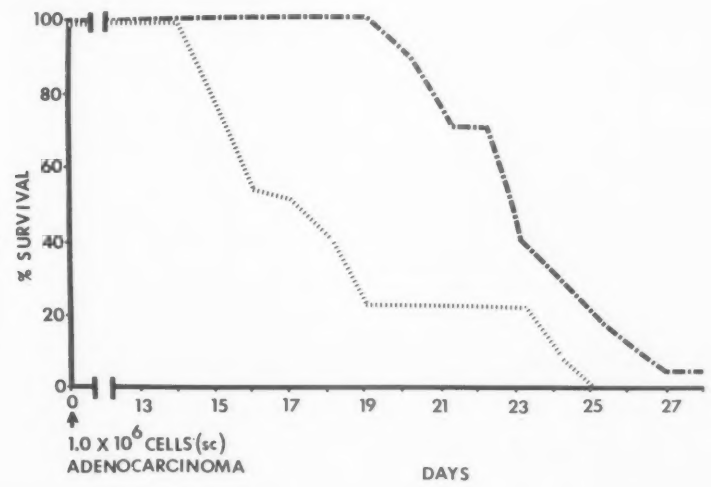

FIGURE 3.—Effect of iv glucan on survival of mice injected sc with 1×10^6 adenocarcinoma cells. Glucan (▪▪▪▪) was injected (0.45 mg/mouse) on Days 0, 3, 6, 9, 12, 15, and 18; total No. = 14. Saline control groups (▪▪▪▪) received isovolumetric saline; total No. = 13.

TABLE 4.—Effect of glucan on growth of 15091A anaplastic mammary carcinoma in A/J mice*

Group	No. of mice	Tumor weight (g)
Saline	17	1.38 ± 0.14
Glucan	19	0.71 ± 0.06†

*All mice received an sc injection of 1×10^7 anaplastic carcinoma cells. Glucan was administered on Days 0, 2, 4, 6, and 8 following tumor challenge at a dose of 0.3 mg/mouse. Controls received isovolumetric saline. Tumors were excised on Day 11.
†Significant ($P < 0.01$).

Effect of Glucan on Tumor Growth and Survival Time of A/J Mice With Anaplastic 15091A Mammary Carcinoma

Subcutaneous tumors were excised and weights were determined in glucan- or saline-treated A/J mice 11 days after administration of 1.0×10^7 tumor cells (table 4). Mice receiving iv injections of glucan (0.3 mg/mouse) at 2-day intervals up to 8 days following tumor cell challenge exhibited a significant reduction in tumor weight relative to the control group. As with the other syngeneic tumors, the antitumor action of glucan was denoted by the approximate 70% decrease of the excised tumor weights.

Of all the syngeneic tumors investigated, the employment of glucan as a posttreatment modality was most efficacious in maintaining survival of mice with the anaplastic carcinoma (fig 5). Mice receiving glucan iv on Days 0, 2, 4, 6, and 8 following sc tumor challenge exhibited only a 20% death rate after 40 days. This is in marked contrast to the 100% death rate of mice receiving isovolumetric saline during an equivalent time period.

Glucan Modification of Lymphocytic (AKR) Leukemia

To further evaluate the effectiveness of glucan against syngeneic tumors, studies were initiated employing AKR lymphocytic leukemia cells. In contrast to our studies with solid tumors, the cultured leukemia cells were administered iv, thus inducing highly disseminated lesions. An injected dose of 1×10^6 AKR leukemia cells was administered on Day 0 followed by glucan injections (0.3 mg/mouse) or isovolumetric saline on Days 1, 2, 4, and 6 (fig 6). These initial studies revealed that this dose of glucan was ineffective in modifying survival as denoted by the 100% death rate in both experimental and control groups by Day 16. The dose of glucan was subsequently increased from 0.3 to 0.45 mg/mouse (fig 6). The administration of a higher dose of glucan on Days 1, 2, 5, and 7 following an iv challenge of 1×10^6 leukemia cells prolonged median survival time to approximately 18 days, compared to 13 days in the control group. In view of the relatively large tumor cell burden employed in these experiments, subsequent studies were undertaken in which the mice were challenged with a lower dose of leukemia cells (fig 7). The administration of

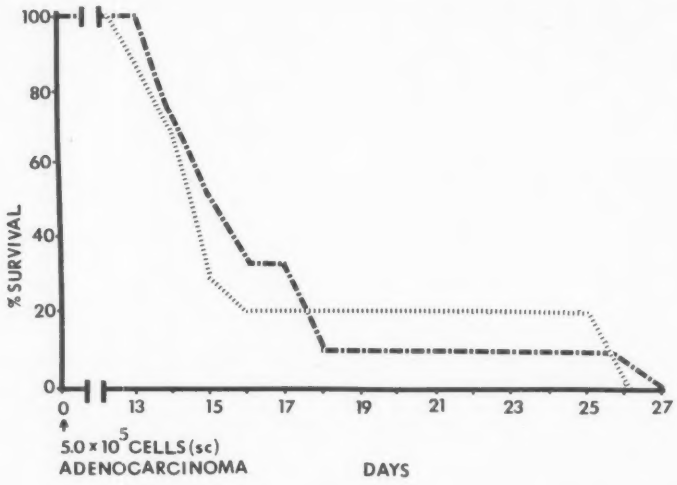

FIGURE 4.—Inability of ip injections of glucan to modify survival of mice injected sc with 5×10^5 adenocarcinoma cells. Glucan (—•—) was administered ip (1 mg) on Days 0, 1, 6, 10, and 15 following tumor cell challenge; total No. = 12. Saline controls (.....) received isovolumetric saline; total No. = 12.

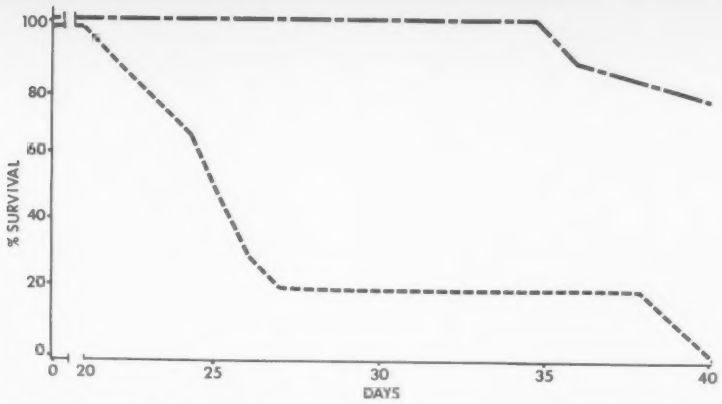

FIGURE 5.—Survival of AJ mice after sc administration of 1×10^7 anaplastic carcinoma cells. Glucan(— — —)was administered iv (0.3 mg/mouse) on Days 0, 2, 4, 6, and 8 following tumor challenge. Saline controls(— — —)received isovolumetric saline; total No. = 10/group.

glucan (0.45 mg/mouse) at 2-day intervals proved to be more effective in the reduced tumor burden mice because the glucan-treated mice manifested a 45% survival after 30 days in contrast to 10% survival in the control group.

DISCUSSION

It is evident, based upon previous preliminary clinical and extensive experimental studies, that glucan provides a unique means to initiate enhanced host resistance to malignancy (9,14). The administration of glucan resulted in both an increase in activation and proliferation of fixed macrophage populations (7,10). The granulomatous tissue reaction induced by glucan as a result of hyperfunction and proliferation of lymphoreticular elements is of a reversible nature and will regress within approximately 2 weeks after cessation of the glucan injection (10). Glucan also enhances cellular (11) and humoral immunocompetence (12) and activates both the classic and alternate pathways of the complement cascade (20). The marked leukopoiesis and proliferation of tissue macrophage elements following glucan administration may be, in

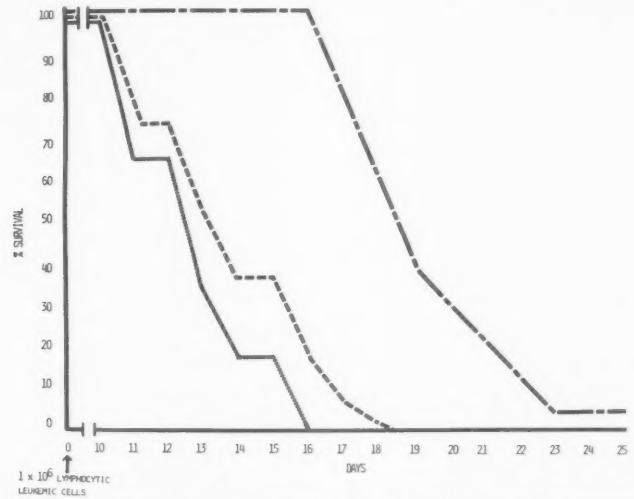

FIGURE 6.—Effect of glucan on survival of AKR mice that received an iv injection of 1×10^6 cultured AKR lymphocytic leukemia cells (——). Glucan was administered at a dose of 0.3 mg/mouse on Days 1, 2, 4, and 6 following tumor cell challenge (— — —); total No. = 6. Glucan was also injected at a dose of 0.45 mg/mouse on Days 1, 2, 5, and 7 (— — —); total No. = 10. Saline controls received isovolumetric saline; total No. = 10.

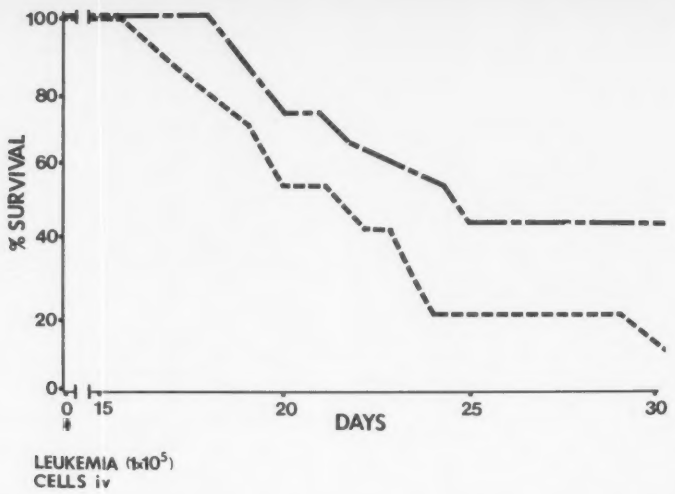

FIGURE 7.—Effect of glucan on survival of AKR mice with lower leukemia cell burden (1 × 10^5/mouse). Glucan (——) was administered iv (0.45 mg/mouse) on Days 1, 2, 5, and 7 and subsequently at 2-day intervals. Saline control mice (– – –) received isovolumetric saline; total No. = 30/group.

part, mediated by increased production of colony-stimulatory factors (21). The ability of glucan to induce an expanded hyperfunctional macrophage population and its capacity to modulate humoral and cellular immunity would be anticipated to contribute to its antineoplastic activity.

The present study extends the utility of glucan as a potential therapeutic agent as denoted by the significant antitumor activity in four syngeneic tumor models. The antitumor activity of glucan can be further appreciated when one considers the diverse characteristics and pathogenesis of the spontaneous murine malignancies which were employed (table 1). Our observations, however, denote a significant variation in responsiveness of the syngeneic tumor models to glucan. Glucan was most effective in maintaining survival of A/J mice with the anaplastic mammary carcinoma and least effective in prolonging survival of AKR mice with lymphocytic leukemia. Such variation in tumor responsiveness has been demonstrated with other commonly employed immunostimulants such as *C. parvum*. For example, Roumiantzeff et al (6) evaluated the efficacy of *C. parvum* in mediating antitumor activity against three tumor models: the nonsyngeneic Ehrlich ascites, the syngeneic AKR leukemia, and the syngeneic YC8 ascitic tumor. The pretreatment regimen of *C. parvum* was only effective in modifying the course of YC8 and Ehrlich ascitic tumors, whereas the immunostimulant was ineffective in modifying survival of AKR mice inoculated with 10^3 leukemia cells (6).

Recently, it was reported that glucan administration had no significant influence on tumor growth or death rate in a variety of syngeneic tumor models (16). Our studies employed two identical tumor models, namely B16 melanoma and 15091A adenocarcinoma. The results of the studies of Hunter et al (16) are difficult to interpret in view of the two different preparations of glucan employed, one of which was prepared in this laboratory. In only one instance was it possible to identify which preparation of glucan was utilized. In none of the studies which were undertaken was it indicated that the dose of glucan and the time of administration were such that macrophage activation was achieved. We have found that doses of glucan such as 0.1 mg/mouse had no inhibitory effect on the growth of the B16 melanoma tumor and did not produce macrophage activation as denoted by enhanced phagocytic function or elevated serum lysozyme activity. The effectiveness of iv glucan in other models, such as the guinea pig hepatoma, remains to be established when appropriate dose-response studies have been undertaken.

We have found that in the acute monocytic leukemia rat model the intralesional administration of glucan was ineffective in modifying tumor growth, while iv administration or the simultaneous administration of glucan with tumor cells was effective in inhibiting tumor growth. Since glucan may enhance macrophage populations by increasing colony-stimulating activity (21) and a delay time of several days occurs before a detectable enhancement in macro-

phage number is apparent, it appears that the failure of glucan to inhibit tumor growth in mice or rats when given intralesionally is due to ineffective macrophage proliferation and activation. Additionally, the sc injection of glucan in rats or mice produces a granulomatous pouch in contrast to an acute inflammatory response in man (13,15).

The route of injection of glucan appears to be an important factor in expressing its antitumor activity. Ip injection was ineffective in modifying the mortality pattern of mice with adenocarcinoma. The antitumor activity of other immunostimulants such as BCG (2) and *C. parvum* (4) also differs depending upon the route of injection and the type of malignancy. Mazurek et al (4) observed that a protective effect of *C. parvum* was obtained against lymphosarcoma when both tumor cells and *C. parvum* were administered iv. In contrast, protection was afforded to mammary carcinoma only when both were administered ip. In either case, the growth of sc injected tumor cells was not altered when *C. parvum* was injected iv. Since the iv injection of mice with *C. parvum* at doses approximating the dose of glucan employed in the present studies was shown to induce intravascular coagulation with consequent hepatic necrosis (22), it is possible that the absence of protection may be mediated by such changes. Neither intravascular coagulation nor alterations in hepatic function were observed in glucan-treated animals. Intralesional injections of glucan have also been previously observed to promote a reduction in tumor size in various human tumors (13,15). It is possible that local mechanisms controlling antitumor activity may differ from the systemic antitumor activity of immunostimulants as postulated by Mazurek et al (4). The decreased growth of localized tumors, inhibition of metastasis, and extended survival of mice with syngeneic tumors following iv administration of glucan may reflect a nonspecific hyperfunction of the reticuloendothelial system.

It is apparent that if glucan is to be employed clinically, it must be effective as an adjunct with accepted modalities of therapy. Our studies have demonstrated the compatibility of glucan immunostimulation with surgery. Survival was enhanced in mice with melanoma following conjoint surgical resection and glucan as compared to either treatment alone. Reduction of tumor burden by the potentiation of host-mediated immune response therefore, may allow more efficient elimination of residual malignancy. The most advantageous time of glucan administration in conjunction with surgical procedures to obtain an optimal therapeutic effect, however, remains to be fully established. It is also possible that glucan will prove to be effective if used with other conventional types of therapy. Recent studies, for example, have demonstrated that conjoint chemotherapy with cyclophosphamide and immunostimulation with glucan is effective in inhibiting metastases and prolonging survival of rats with acute myelogenous leukemia (23).

The immunopotentiating effect of glucan is also denoted by a synergistic enhancement of survival and suppression of hepatic metastasis in leukemic rats following concurrent administration of glucan and glutaraldehyde-fixed tumor cells (18). Additionally, suppression of pulmonary metastasis has been observed in mice with B16 melanoma when glucan was injected in combination with irradiated tumor cells (24). Indeed, facilitation of metastasis was observed in this study when mice were treated with certain doses of levamisole or BCG. Such facilitation was not observed with glucan (24).

Glucan has also been observed to increase serum lysozyme, in association with enhancement in phagocytic function. In addition, glucan-treated mice showed enhanced resistance to *Staphylococcus aureus* (25), denoting that glucan induced an enhanced state of host resistance against certain bacterial infections as well as neoplasia. Since infectious complications can be significant problems in patients with malignancies, the duality of therapeutic effects of glucan is a most positive feature.

The present composite studies conducted with four syngeneic tumor models, coupled with previous clinical observations, denote a potential for glucan in immunotherapy and immunoprophylaxis. Additionally, delineation of the mechanisms of antitumor activity of glucan may define the immunophysiologic events which are associated with the modification of the malignant state.

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Preliminary Observations on the Effect of Glucan in Combination With Radiation and Chemotherapy in Four Murine Tumors [1]

C. C. Stewart,* F. A. Valeriote, and C. A. Perez [2,3]

SUMMARY

The efficacy of glucan in combination with local radiation therapy was measured using three solid murine tumors of differing abilities to induce a host defense. Using the KHT fibrosarcoma which induces no measurable host defense, glucan did not improve tumor-free survival over radiation alone; the combination produced a marginal improvement in tumor-free survival in animals bearing the highly immunogenic EMT-6 tumor. The most marked improvement in tumor-free survival was found with the mildly immunogenic 6C3HED lymphosarcoma. The efficacy of glucan in combination with BCNU chemotherapy was measured using the disseminated AKR transplantable leukemia; the combination yielded a high level of cures compared to no survival for either agent alone. Using the AKR transplantable leukemia in an F_1 model, the effect of amphotericin B (AmB) alone or in combination with BCNU was tested. AmB or BCNU alone had little or no curative effect when tested in (AKR × DBA)F_1 mice, but 56% of mice were cured when combined therapy was employed. When tested in (AKR × C57BL)F_1 or (AKR × A)F_1 mice, a small fraction was cured with AmB alone while about 90% were cured with either BCNU alone or the combination.

[Cancer Treat Rep 62:1867–1872, 1978]

Several investigators have demonstrated that the β1,3 polyglucose agent, glucan, induces activation of the mononuclear phagocyte system. Upon iv injection, major reticuloendothelial tissues hypertrophy (1–4), and there is accelerated intravascular clearance (5,6), augmented primary and secondary antibody responses (7), and enhanced allogeneic transplant rejection (8,9). Glucan thus appears to have properties similar to those of an immunoadjuvant.

Glucan has also been shown to have antitumor properties, apparently as a result of its action on host-defense mechanisms to cancer. Increased tumor-free survival was observed for animals bearing the Shay myelogenous leukemia (10,11) and B16 melanoma (10) when treated with glucan. This agent has also been shown to be effective in the treatment of some human malignancies (12,13).

In this study we sought to determine the effect of glucan in combination with radiation or chemotherapy on four tumor models selected on the basis of their relative ability to induce a host defense. As previously described (14), the EMT-6 tumor appears to be the most immunogenic, the 6C3HED lymphosarcoma appears to be mildly immunogenic, and the KHT fibrosarcoma shows no ability to induce a host defense. Each of these is a solid localized tumor. The AKR leukemia was selected as a disseminated neoplasm.

MATERIALS AND METHODS

Tumors and Mice

The KHT fibrosarcoma and 6C3HED lymphosarcoma are syngeneic with C3H mice, the EMT-6

[1]Supported by Public Health Service grant CA-13053 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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[3]We thank Barbara Hente and Sandra Tolen for their expert technical assistance.

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tumor is syngeneic with BALB/cKa mice, and the AKR leukemia is syngeneic with AKR mice. The solid tumors have been maintained by sc injection of 10^6 viable tumor cells in 0.1 ml in the flank of appropriate mice every 3 weeks. Donor mice used for both passage and experiments were established from tumors dissected from the flank of mice, minced in alpha-MEM medium supplemented with 20% fetal calf serum and 20 mM morpholinosulphonic acid (MOPS), and, except for the EMT-6 tumor, passed through a 120-mesh stainless steel screen. The EMT-6 tumor was trypsinized for 1 hour in 50 ml of 0.05% trypsin before passage through the screen. Cell suspensions were centrifuged at 200 × *g* and resuspended in medium, and after counting the cells, the concentration was appropriately adjusted for injection.

The AKR leukemia (15) was transplanted weekly by iv injection of 2×10^5 cells derived from the spleens of tumor-bearing mice. For the experiments described here, 10^6 leukemia cells were injected iv into the experimental mice.

F_1 hybrid mice were prepared by mating female AKR/J mice with the appropriate male strain. The combinations AKR/J × C3H/HeJ, AKR/J × DBA/2J, and AKR/J × C57BL/6J were produced.

Tumor Treatment

When tumors became palpable (2–4 mm in diameter), the tumor-bearing flank was locally irradiated as previously described (14) using a special lead block to shield the rest of the animal (whole-body exposure limited to 1% of the total tumor dose). Radiation was administered using a 200-kVp X-ray machine (15 ma, 2 mm copper half-value layer, 20 cm TSD) at approximately 400 rads/minute. The KHT fibrosarcoma was treated with 4000 rads while the EMT-6 and 6C3HED tumors were treated with 3000 rads as a single dose. These doses were found to be curative in approximately 30% of mice (14). The cure rate and dose were chosen in order to distinguish improvement in tumor-free survival with glucan as compared to radiation alone.

Glucan was supplied by Dr. N. R. Di Luzio as a suspension in saline containing 35 mg/ml. Glucan was stored at 4°C and the same batch was used for all experiments. BCNU was obtained from the National Cancer Institute, Bethesda, Md.

RESULTS

Growth and Antitumor Reactivity

The tumors have been selected on the basis of their ability to elicit a host defense. A summary of the parameters used to identify the tumor's growth characteristics and the relative host defense is shown in table 1. The EMT-6 is the only tumor in which mice can be pre-immunized. Other than pre-immunization with killed tumor cells, the 6C3HED lymphosarcoma shares the other host defense features of the EMT-6 tumor. The AKR leukemia appears to induce a mild host defense since cured mice will resist a challenge injection (16). The KHT fibrosarcoma is the least able to induce a host defense.

TABLE 1.—Comparison of growth and host antitumor reactivity

	Tumor			
	EMT-6	6C3HED	KHT	AKR
Growth parameter measured				
TD50* (cells/mouse)	10^2–10^3	10^1–10^2	10^1–10^2	10^0–10^1
Palpable size† (No. of cells)	1.5×10^6	2×10^7	3.4×10^6	—
Doubling time† (in vivo)	48 hrs	12 hrs	36 hrs	9 hrs
Plating efficiency (in vitro)	29%	0	1%	0
Defense parameter measured				
Immunization with killed cells	Yes	No	No	No
Concomitant immunity	Yes	Yes	No	‡
Cured mice resist challenge	Yes	Yes	No	Yes
Infiltrated with lymphocytes and macrophages	Yes	Yes	Yes	‡
Syngeneic mouse strain	BALB/cKa	C3H	C3H	AKR

* No. of cells required to produce tumors in 50% of the mice.
† Determined from endpoint dilution assay.
‡ Not measured due to disseminated nature of the tumor.

Effect of Glucan in Combination With Radiotherapy

Mice were injected with 10^6 tumor cells in the flank. When the tumor became 2–4 mm in diameter (Day 3 for 6C3HED lymphosarcoma, Day 5 for KHT fibrosarcoma, and Day 6 for EMT-6 tumor), the tumor was locally irradiated. Animals were then randomized into either a group which received no further treatment or a group which received 1 mg of glucan iv twice weekly for 28 days. The first injection of glucan was given 4 hours after irradiation of the tumor.

The results of these studies are shown in figure 1 where the tumor-free survival is plotted as a function of the time after tumor cell inoculum. In figure 1A it can be seen that glucan was not effective in controlling the KHT fibrosarcoma; the increase in lifespan was due solely to radiation alone. In fact, there was some suggestion that glucan in combination with radiation was less effective than radiation alone in improving lifespan of these tumor-bearing mice.

In figure 1B, mice harboring the EMT-6 tumor demonstrated a marginal improvement in survival when treated with glucan plus radiation. The most impressive results were obtained using the 6C3HED lymphosarcoma (fig 1C) in which a significant increase in tumor-free survival was found following treatment with glucan plus radiation.

Effect of Glucan in Combination With BCNU Using the Transplantable AKR Leukemia

In these studies, groups of ten AKR mice received 10^6 leukemia cells, with glucan administered at different times relative to the leukemia cell inoculation. BCNU was always administered 4 days after the leukemia cells. The dose of BCNU chosen, 0.2 mg/mouse, was that found to be effective in our chemoimmunotherapy model (16). This dose yields a surviving fraction of about 10^{-2} with a concomitant 2-day increase in lifespan. The results are shown in table 2. Treatment of leukemic mice with glucan only had no effect on lifespan. Glucan was also ineffective in combination with BCNU when it was injected 14 days before the leukemia cells. However, when injected at 7 days or 1 day before or 1 or 2 days after leukemia cells, there was a 5%–15% cure rate even though there was no change in the median survival time of the animals that died. When glucan was injected on Days 3 or 4 after the AKR leukemia cells, there was a consistent improvement in the cure rate as well as the median survival time of animals that died. A 4-day schedule of glucan was also examined since it has been found to be most effective in the AKR system for another immune adjuvant (17); it was not as effective here as the single-dose schedule.

Effect of Amphotericin B (AmB) in Combination With BCNU in F_1 Tumor-Bearing Mice

We have attributed the curative response found with the AmB-BCNU combination against AKR leukemia to a stimulation of the immune system by the AmB, with the BCNU providing sufficient cell killing to produce a challenge to the "activated" immune system by the killed cells (16,17). We therefore expected that an F_1 rather than a syngeneic host might provide a test system in which we could observe a response with the immune-active agent alone. Leukemia cells (10^6) were injected into recipient mice, with AmB (0.5 mg/mouse) administered ip on Days 1–4; 0.2 mg of BCNU was injected on Day 4. Groups of five or ten mice received either agent alone or the combination. The results of four or five separate experiments for the various hybrids tested are shown in table 3.

For the (AKR × DBA)F_1 study (table 3), AmB had no effect on either the median survival time or the cure rate (equivalent to the 30-day survival rate). While BCNU alone improved median survival time as expected, AmB in combination with BCNU yielded a large fraction of long-term survivors in contrast to that found with BCNU alone. This result was essentially identical to that found in the syngeneic AKR model for AmB plus BCNU (16).

For the (AKR × C57BL/6)F_1 study (table 3), a small fraction of mice were cured with AmB alone and a larger fraction with BCNU alone. The addition of AmB to BCNU, however, did not significantly augment the cure rate of mice over BCNU alone.

Finally, for the (AKR × A)F_1 system, AmB alone produced a significant cure rate as did BCNU alone; the AmB-BCNU combination led to nearly total cures in this group of mice.

DISCUSSION

We believe the preliminary results of this study suggest that glucan may be an effective agent for treatment of some tumors in combination with radiotherapy and/or chemotherapy. It seems that glucan is as effective as other adjuvants presently being tested but it has the advantage of being nontoxic to the animal since its metabolic by-product is glucose.

The agent appeared to be most useful against tumors which are mildly immunogenic (Gardner lymphosarcoma and AKR leukemia). This could be

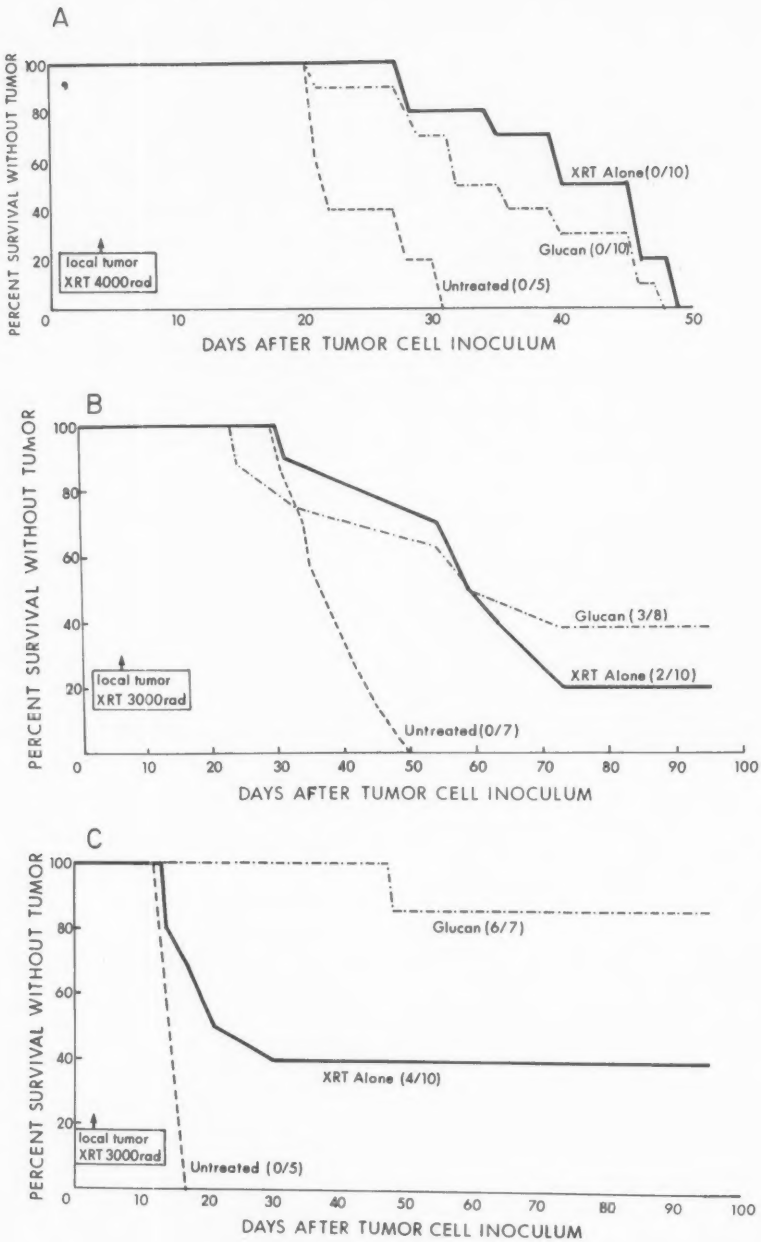

FIGURE 1.—Survival of mice bearing (A) KHT fibrosarcoma, (B) EMT-6 tumor, or (C) Gardner lymphosarcoma either untreated, treated with radiation therapy only, or treated with glucan in combination with the radiation.

TABLE 2.—Effect of glucan in combination with BCNU

	Median survival time (days)	Survival at 30 days
Control	5.5	0
Glucan alone	5.5	0
BCNU alone	7.5	0
Glucan on Day −14 plus BCNU	7.5	0
Glucan on Day −7 plus BCNU	7.5	10%
Glucan on Day −1 plus BCNU	7.5	5%
Glucan on Day + 1 plus BCNU	7.5	15%
Glucan on Day + 2 plus BCNU	8.0	10%
Glucan on Day + 3 plus BCNU	12.0	40%
Glucan on Day + 4 plus BCNU	9.0	30%
Glucan on Days + 1, 2, 3, and 4 plus BCNU	12.5	0

TABLE 3.—Effect of AmB, BCNU, or the combination on (AKR)F_1 mice

	Median survival time (days)	Survival at 30 days*
AKR × DBA		
Control	5.5	0 (0/20)
AmB	5.5	0 (0/20)
BCNU	7.5	5% (1/20)
AmB + BCNU	†	56% (19/34)
AKR × C57BL/6		
Control	5.5	0 (0/20)
AmB	5.5	5% (1/20)
BCNU	†	89% (17/19)
AmB + BCNU	†	88% (22/25)
AKR × A		
Control	6.0	8% (2/25)
AmB	7.0	36% (9/25)
BCNU	†	88% (22/25)
AmB + BCNU	†	93% (28/30)

*Nos. in parentheses = No. of 30-day survivors/total in the experimental group.

†Not calculated since > 50% of the animals survived.

interpreted to mean that animals which mount a good host defense against their tumor cannot be further augmented, while animals whose host defense is only mildly stimulated can be further amplified by the addition of an immunoadjuvant. Glucan appears to be a good immunoadjuvant in this regard.

Utilizing the F_1 model, we have shown that AmB itself can produce long-term cures in some F_1 hybrid combinations harboring an advanced tumor. The mechanism for this antitumor activity is unknown but may involve concepts of hybrid resistance. Rejection of spontaneous AKR lymphoma in F_1 hybrids is being studied by others to define the underlying (cellular) mechanism of action and relate the phenomena of allogeneic inhibition and hybrid resistance (18). Such results suggest that resistance (and likely the AmB stimulation of such resistance) is probably mediated by cytotoxic T cells as evidenced by the susceptibility of cytocidal activity to anti-thy 1.1 serum. An important point is that a host defense can be boosted to a level capable of eliminating tens of millions of leukemia cells and this might provide an appropriate model for screening of adjuvants. While it might be argued that the F_1 represents a poor model because of possible allogeneic effects unrelated to the true host-tumor response, two points are important: first, the mechanisms of the response and its activation may be identical to that obtained in the autochthonous host but, in the hybrid, a stronger response develops; second, in terms of animal-tumor models, most have been passaged for many generations, and the recipients have been bred for many generations. Therefore, the possibility of deviants makes any "syngeneic" system also suspect. Thus, the F_1 model using various strain combinations offers a system in which the cellular effects of immune adjuvants can be studied in greater detail. Quite possibly the mechanism is identical to the hybrid resistance noted for hematopoietic cells which may also involve macrophages (19).

In conclusion, glucan and AmB appear to be as effective as any of the presently reported immunoadjuvants which are being intensely studied both in the clinic as well as in the laboratory animal models. Glucan has the advantage of limited toxicity and, therefore, a potentially greater patient acceptability.

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Effect of Glucan and Other Adjuvants on the Clearance of Radiolabeled Tumor Cells From Mouse Lungs [1]

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SUMMARY

The iv injection of two forms of glucan (DiLuzio and Yamamura), *Corynebacterium parvum*, or bacillus Calmette-Guérin (BCG) caused a significant increase in the rate and extent of clearance of subsequently injected radiolabeled T1699 carcinoma cells from the lungs of syngeneic DBA_2 mice. Dose-response curves were obtained for each agent, the relative rates of clearance of radiolabeled cells were established, and preliminary sequential comparative studies were undertaken. The optimum dose for *C. parvum* appeared to be lower than for DiLuzio glucan which appeared to be lower than for Yamamura glucan or BCG. The results indicated that at optimum doses one form of glucan (DiLuzio) was as effective as *C. parvum* and more effective than BCG or a second form of glucan (Yamamura), with respect to the time for the genesis, the magnitude, and the length of time of the response. Very preliminary experiments with a highly malignant and poorly immunogenic cell line of B16 melanoma in syngeneic C57BL/6J mice demonstrated a correlation between an increased level of clearance of radiolabeled cells and a reduced number of subsequent macroscopic lung tumor nodules.

[Cancer Treat Rep 62:1873–1880, 1978]

The large-scale use of reticuloendothelial (RE) stimulants in cancer patients followed earlier reports of their antitumor activity in animal models. The majority of these data were obtained by immunoprophylaxis against a subsequent local challenge with transplanted tumors (1,2). The results of more relevant experiments in which the RE stimulants were given after an inoculum of tumor cells have proved less impressive (1–3), and those involving spontaneous metastases (2,4–8), the most relevant of all such studies, have not often been reported (1,3). Important features of these experiments such as the variation due to routes of administration (2,9) and dose dependence (1–3,8,10) have apparently not been accorded their due significance in the clinic, particularly with respect to the frequent reports of tumor promotion (2,3,10–12) and facilitation of metastases (2,5,8).

The most commonly used agents in man are bacterial and are frequently associated with unpleasant side effects, although transient in nature. In the case of bacillus Calmette-Guérin (BCG), systemic infections or multiple granulomatosis has been reported in a significant number of patients (13). All of the bacterial agents are potent immunogens and their repeated use in immunocompetent patients leads to increasingly vigorous reactions against the bacterial antigens.

There is a small risk of catastrophic allergic phenomena including anaphylaxis (ref 13 and Proctor JW, personal observation), and the deaths of two patients have been reported as a result of Schwarzman reactions (14). These are most likely to occur following intralesional injection of the agent. Cur-

[1]Supported by Health Research and Services Foundation grant T-26 and by Public Health Service grant CA-10438 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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[4]We thank Dr. N. R. DiLuzio for supplying glucan, DiAnna Gaydos for technical assistance, and Linda Lira for typing the manuscript.

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rently, however, perhaps the most worthwhile use for such agents has been to tackle the difficult problem of multiple local dermal metastases in melanoma patients who are otherwise apparently disease-free. It should be recognized, however, that this is a rare form of cancer.

There is a pressing need for further studies of alternative nonimmunogenic agents with no infective properties and a low potential for toxicity. Such agents should also be suitable for systemic administration and have as nearly as possible an antitumor potential equivalent to that of the currently used preparations. Two of the most promising agents are glucan (15–17), a yeast cell-wall component (18), and pyran copolymer (19,20). Others are also being investigated, and there is an increasing need for a practical method of comparing their antitumor activities as they are discovered. Obviously, studies in man will form the basis of any final decisions, but these are not likely to be more than marginally helpful at the present moment, and only animal studies will achieve readily analyzable results in a reasonable period of time.

Of the many approaches available, the characteristics of spontaneously metastasizing models (2,4–8) most nearly approach the clinical problem. However, these are difficult to manage and to reproduce. They require large numbers in each group, and for comparisons of a number of doses of several agents they are totally impractical for preliminary screening. Because immunoprophylaxis is easier to achieve than immunotherapy, models in which lung tumors form from intravenous tumor cells (1–3) have some clinical relevance and can be fairly easily managed.

However, the range of numbers of lung tumor nodules is often quite great (range, 2–100) for groups of animals given identical injections of tumor cells, requiring significantly large numbers of animals for each group. Furthermore, the counting of macroscopic tumor nodules is highly subjective and is only tenable in view of the lack of better methods that are not both tedious and time-consuming. We have taken a more practical approach which allows the use of comparably smaller numbers of animals in each experimental group and permits comparison of several doses of more than one agent in each experiment.

This method (measurement of the clearance of iv injected radiolabeled tumor cells from the lungs of syngeneic rodents) has been used previously to monitor the distribution and fate of blood-borne cells in specifically (21–24) and nonspecifically (25) immune animals. Our preliminary results suggest that it may prove to be a practical bioassay for screening antitumor agents of the RE stimulant class in the future.

MATERIALS AND METHODS

Animals.—Syngeneic female DBA_2 and male C57BL/6J mice (6–8 weeks of age) were obtained from Jackson Laboratories, Bar Harbor, Me, and were maintained on mouse chow and tap water ad libitum in a clean environment.

Tumors.—Mechanically derived suspensions of the T1699 mammary adenocarcinoma and of the B16 melanoma, which arose in the DBA_2 and C57BL/6J strains of mice respectively, were maintained in vivo, in liquid nitrogen, or in short-term tissue culture. The T1699 carcinoma, which is highly immunogenic and which metastasizes readily (although these secondary deposits regress spontaneously), was used in the initial comparative studies. The B16 melanoma line, obtained from Jackson Laboratories, has proved to be poorly immunogenic and extremely malignant.

Glucan.—Glucan in a water-insoluble form was obtained from Dr. N. R. DiLuzio, Tulane University, New Orleans, La, and in a hydrolyzed water-soluble form from Dr. Y. Yamamura, Allegheny General Hospital, Pittsburgh, Pa.

BCG.—A Glaxo preparation containing between 5 and 27 $\times$ 10^6 viable organisms/ml was used as a fresh suspension.

Corynebacterium parvum.—The Burroughs Wellcome Co (Research Triangle Park, NC) preparation, a suspension of dead organisms, was used.

Radiolabel.—The thymidine analog, ^{125}IUdR (Amersham Searle, Arlington Heights, Ill), was used in all experiments (specific activity, 0.5–1.50 millicuries/mmol).

Labeling of tumor cells.—Single-cell suspensions of tumor were obtained by enzymatic digestion and were incubated in tissue culture with ^{125}IUdR for 48 hours during the logarithmic growth phase so that approximately 1 cpm/3–10 tumor cells was incorporated (24). Following harvesting and repeated washing, between 5×10^4 and 2×10^5 cells were injected iv by the lateral tail vein.

Analysis of radiolabel loss.—Groups of four to seven mice were exsanguinated between 10 minutes and 7 ½ hours after iv injection and the lungs were removed and counted on a conventional gamma counter for 1 minute. The results were usually expressed graphically as the percentage change in radiolabel loss compared to normal controls as follows:

$$\text{Change in radiolabel loss} = \frac{\text{cpm normal lung} - \text{cpm experimental lung}}{\text{cpm normal lung}} \times 100.$$

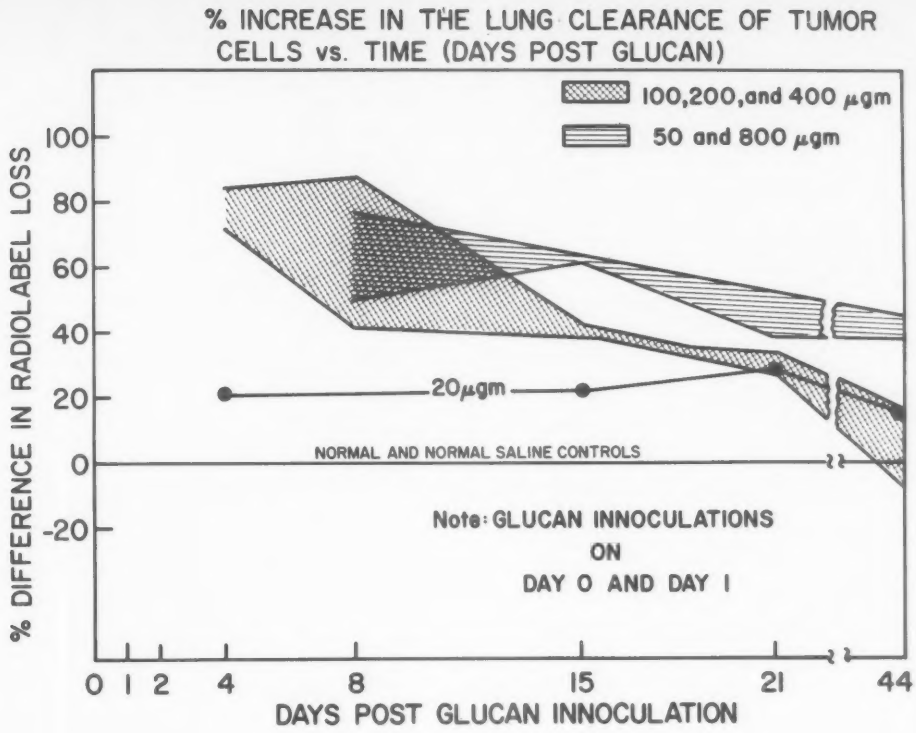


FIGURE 1.—Various doses of glucan (DiLuzio) were injected on Days 0 and 1 and groups of mice were challenged with 5×10^4 ^{125}IUdR-labeled T1699 tumor cells between Days 2 and 44 in a series of 3 separate experiments. The mean values for the % change in the level of lung radioactivity compared to control mice 4 hrs after challenge were combined in all experiments for 50- and 800-μg and for 100–400-μg doses.

All results were analyzed by the Mann-Whitney nonparametric test.

RESULTS

Preliminary Findings With Glucan (Fig 1)

By Day 4, after iv administration of glucan on Days 0 and 1, but not by Day 2, compared to normal and normal saline-treated controls, an increased clearance of radiolabeled tumor cells from the lung was apparent 4 hours after iv tumor cell injection at doses of ≥ 50 μg/mouse. The dose response plateaued above 100 μg up to doses of 800 μg/mouse. The activity peaked between Days 4 and 15 and declined progressively, but more rapidly so for the middle range of doses, so that by Day 44, only mice treated with 50 and 800 μg registered a statistically significant increase in clearance compared to the controls.

Comparative Studies (Dose-Response Curves at Day 8) (Fig 2)

Well-defined dose-response curves were obtained for the four agents tested. The optimum doses for the agents were 25 μg for *C. parvum*, 50 μg for DiLuzio glucan, 100 μg for Yamamura glucan, and 200 μg for BCG. The increased level of clearance of radiolabeled tumor cells was similar for all agents except for the Yamamura glucan-treated mice, in which the effect was smaller than that for any other agent. There was a plateau response for *C. parvum*, DiLuzio glucan, and BCG, and in the case of the Yamamura glucan, the effect of increasing the dose to 200 μg caused a number of deaths from asphyxia, apparently related to the high viscosity of the preparation.

Rates of Clearance of Radiolabeled Tumor Cells After Treatment With Optimal Doses (Fig 3)

There was an increased rate of clearance of

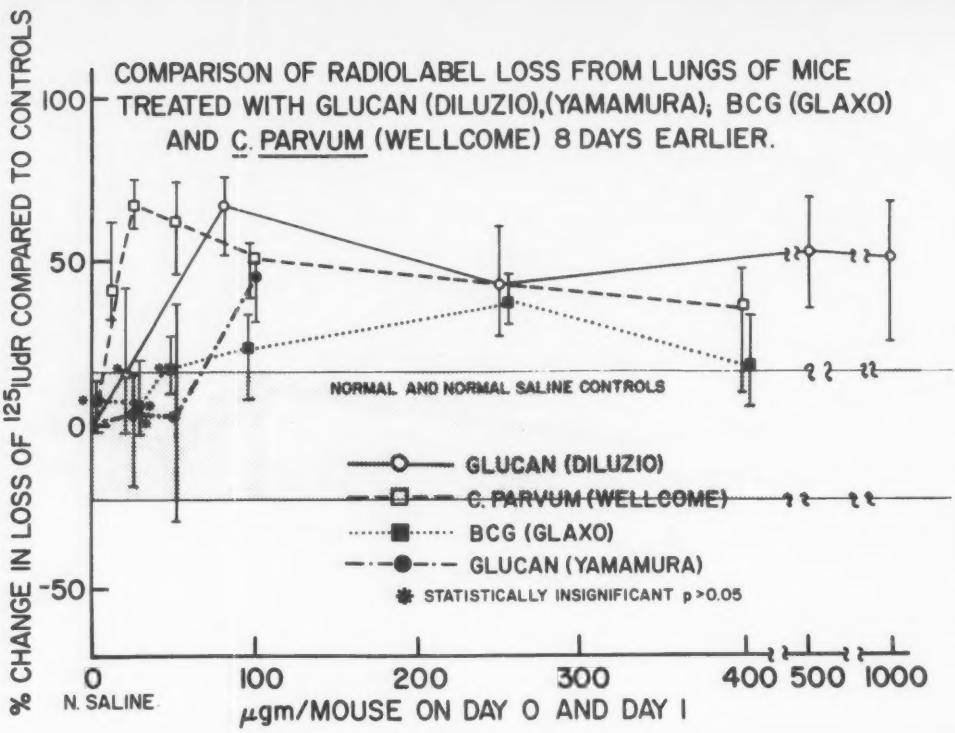


FIGURE 2.—Mice were injected iv with various doses of BCG, 2 forms of glucan (DiLuzio and Yamamura), or *C. parvum* on Days 0 and 1. An iv challenge with 10^5 radiolabeled T1699 tumor cells was given on Day 8 and groups of mice were killed 4 hrs later. The lungs were harvested and counted on a gamma counter.

radiolabeled tumor cells from the lungs of mice treated with all of the agents tested. It was apparent earlier in BCG-treated mice than in those mice treated with the other agents, but there was little difference in the extent of clearance between the agents at 4 hours.

Sequential Studies After Treatment With Optimal Doses (Fig 4a–c)

The initial comparison with the two forms of glucan demonstrated an earlier, more marked, and more prolonged capacity for DiLuzio glucan to cause an increased clearance of radiolabeled tumor cells. The second study demonstrated a fairly equivalent effect for *C. parvum* and DiLuzio glucan with respect to the time of initiation, magnitude, and length of time for an effect on radiolabeled tumor cell clearance. In the final experiment in the series, DiLuzio glucan produced an earlier and longer-lasting effect than did BCG, and the optimum dose for the glucan was lower than that for BCG.

Correlation of the Extent of Radiolabeled Cell Clearance With a Reduced Capacity of Tumor Cells to Grow in the Lung (Table 1)

In a single preliminary experiment, treatment of C57BL/6J mice with 50 or 100 μg of DiLuzio glucan on Days 0 and 1 induced a greater clearance of radiolabeled B16 tumor cells from the lung within 4 hours of tumor cell injection. In these mice, a significant reduction of the number of lung tumor nodules also occurred in the glucan-treated mice.

DISCUSSION

A significant increase in the rate and extent of clearance of iv injected radiolabeled T1699 tumor cells from the lungs of DBA_2 mice resulted from prior iv injection of BCG, *C. parvum*, and two forms of glucan. The findings are reminiscent of those of Bomford and Olivotto (25) with *C. parvum* in another animal model, and of our own with glucan in a syngeneic rat sarcoma system (Proctor JW, personal observations). Dose-response curves suggested

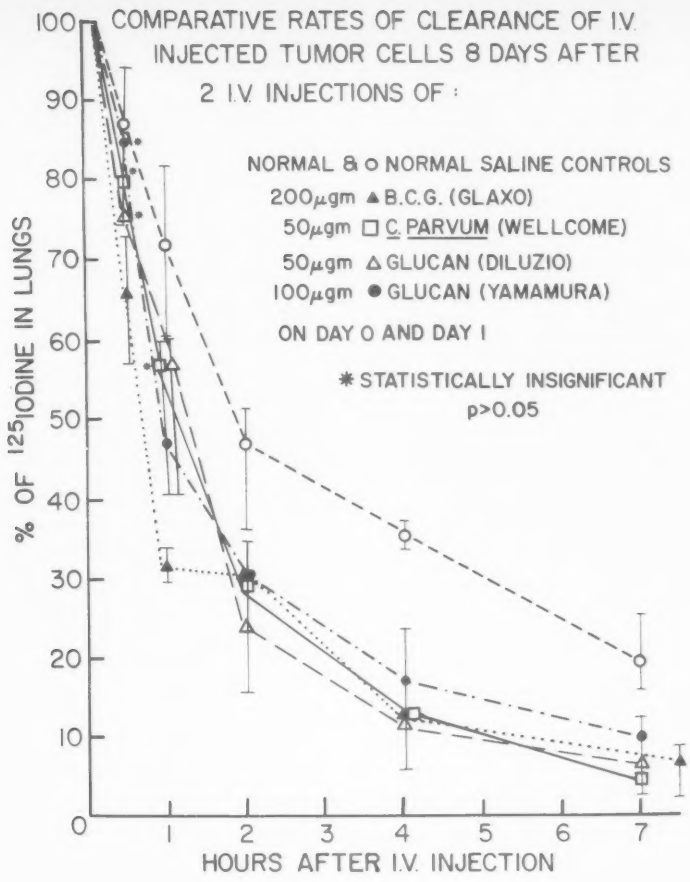


FIGURE 3.—Mice were injected with an approximately optimum dose of either BCG, two forms of glucan, or *C. parvum* on Days 0 and 1, and were challenged on Day 8 with 10^5 radiolabeled T1699 tumor cells. The mice were killed between 10 mins and 7 ½ hrs later and the lungs were harvested and counted on a gamma counter.

that the optimum dose for *C. parvum* (25–50 μg) was lower than that for DiLuzio glucan (50–100 μg), Yamamura glucan (100 μg), or BCG (200 μg). In no instance was a significantly reduced extent of clearance of radiolabeled tumor cells encountered compared to that of control mice. The sequential studies suggested that *C. parvum* and DiLuzio glucan produced earlier, more definitive, and longer-lasting effects on radiolabeled cell clearance than BCG or Yamamura glucan, and reinforced the earlier results relating to the relative optimum doses of tnese agents.

We stress emphatically that the latter results were obtained in single experiments and as a result they must be interpreted with caution. Nevertheless, it can be noted, for the same reason, that the technique is practical. The reproducibility from one experiment to another based on the repeated observations with glucan, while it left a lot to be desired, was not unreasonable for an "in vivo" system. It is evident, however, that truly definitive comparative experiments must be carried out without reference merely to historical data. The T1699 tumor system is syngeneic to DBA_2 mice, which are notoriously susceptible to intercurrent infection, and it is quite possible that this factor alone accounted to a major degree for any lack of reproducibility.

The results taken together support the concept that the radiolabeled cell clearance system might prove to be a practical, rapid, and relatively inexpensive method for comparing the antitumor effects of RE stimulants. Certainly a number of criteria can be established for these agents.

However, the DBA_2 system we used is definitely

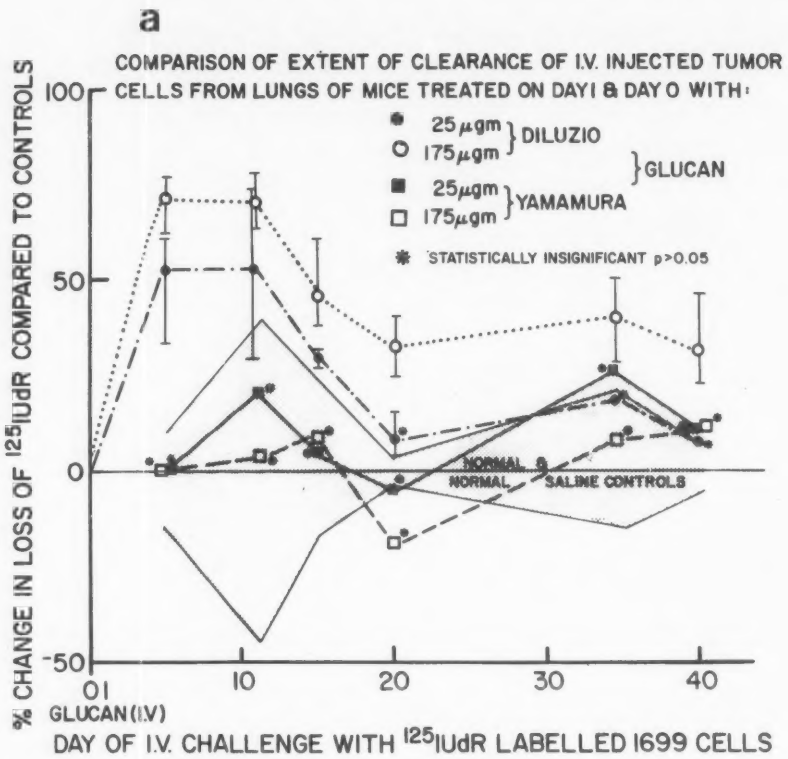
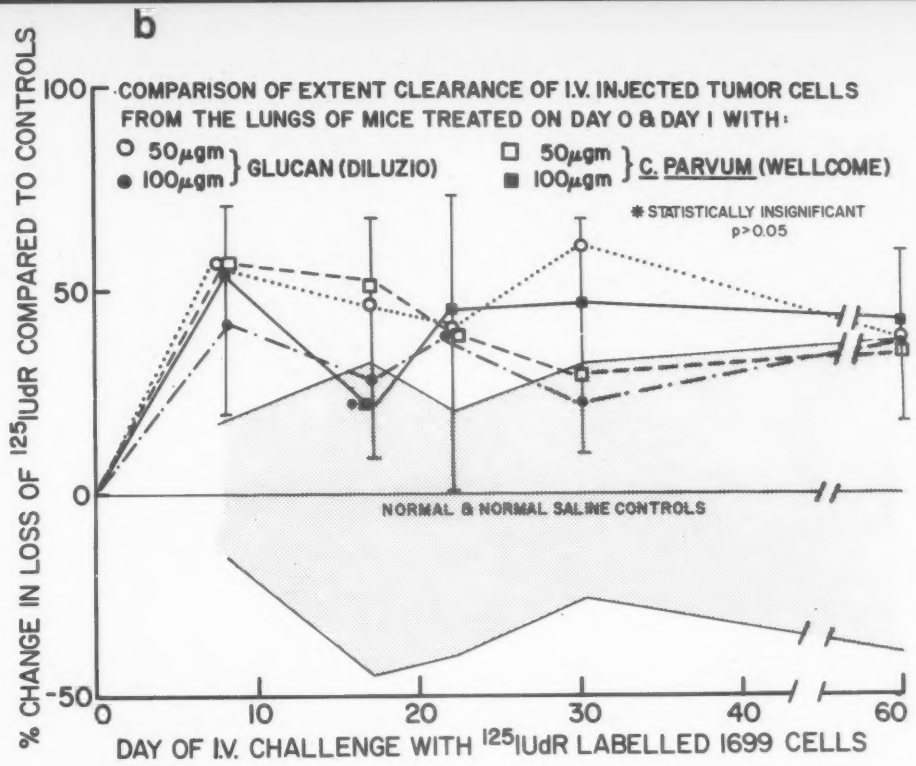


FIGURE 4 (a, b, and c).—Shaded areas = range of values for normal and normal saline-treated control mice. Vertical bars = range of values for the change in lung radioactivity, expressed as a percentage of the mean value for the controls, of all groups of mice in which there was a statistically significant difference compared to the controls at $P < 0.05$ level. Mice were injected iv with 2 doses of either DiLuzio or Yamamura glucan, *C. parvum*, BCG, or normal saline on Days 0 and 1. They were challenged with 10^5 radiolabeled T1699 cells at various time points thereafter and were killed 4 hrs later, and the lungs were harvested and counted on a gamma counter.


b
COMPARISON OF EXTENT CLEARANCE OF I.V. INJECTED TUMOR CELLS FROM THE LUNGS OF MICE TREATED ON DAY 0 & DAY 1 WITH:
○ 50μgm
● 100μgm
GLUCAN (DILUZIO)
□ 50μgm
■ 100μgm
C. PARVUM (WELLCOME)
* STATISTICALLY INSIGNIFICANT p>0.05
NORMAL & NORMAL SALINE CONTROLS
% CHANGE IN LOSS OF 125IUdR COMPARED TO CONTROLS
100
50
0
-50
0
10
20
30
40
60
DAY OF I.V. CHALLENGE WITH 125IUdR LABELLED 1699 CELLS

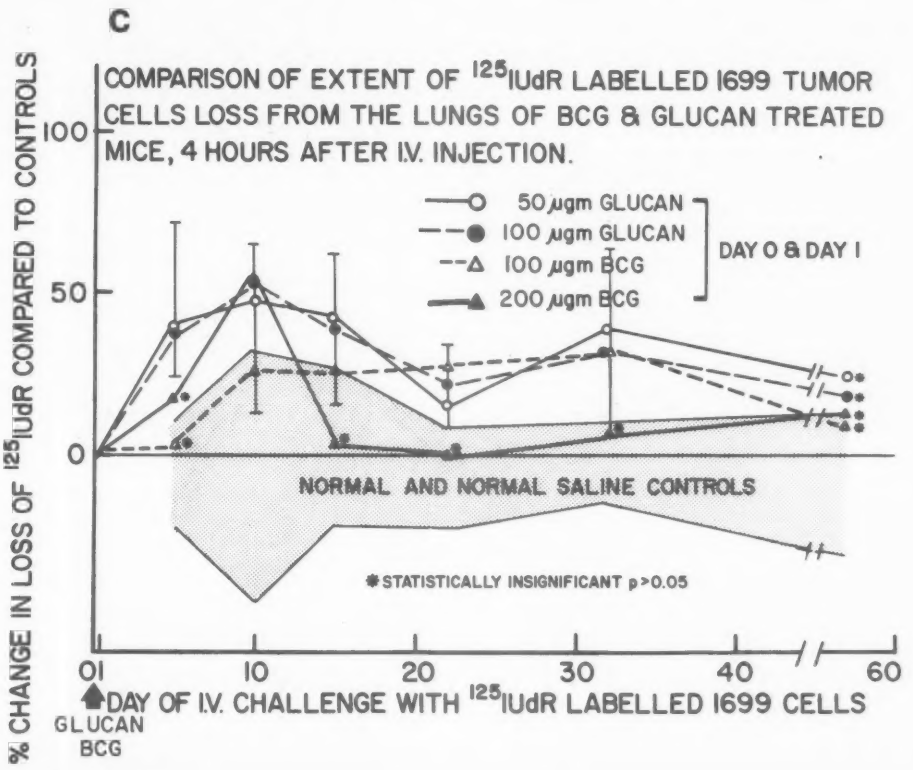

c
COMPARISON OF EXTENT OF 125IUdR LABELLED 1699 TUMOR CELLS LOSS FROM THE LUNGS OF BCG & GLUCAN TREATED MICE, 4 HOURS AFTER I.V. INJECTION.
50 μgm GLUCAN
100 μgm GLUCAN
100 μgm BCG
200 μgm BCG
DAY 0 & DAY 1
NORMAL AND NORMAL SALINE CONTROLS
* STATISTICALLY INSIGNIFICANT p>0.05
% CHANGE IN LOSS OF 125IUdR COMPARED TO CONTROLS
100
50
0
0
10
20
30
40
60
DAY OF I.V. CHALLENGE WITH 125IUdR LABELLED 1699 CELLS
GLUCAN
BCG

TABLE 1.—Radioactivity in the lung 4 hrs after iv challenge with ^{125}IUdR-labeled B16 cells* and subsequent outgrowth of tumor nodules

Treatment iv on Days 0 and 1	cpm/lung 4 hrs after injection (range)	Tumor nodules in lungs	
		Incidence	Mean No. (range)
Normal saline controls	4844 (4446–5312)	4/4	> 75 (51–> 100)
50 μg of glucan	921† (854–956)	3/6	6.9† (0–25)
100 μg of glucan	1773† (1123–2093)	3/7	6.3† (0–24)

*A total of 10^5 B16 cells injected iv on Day 8 registered 13,299 cpm.

†Statistically significant compared to control values at $P < 0.002$ level (Mann-Whitney test).

unsuitable for these purposes, since macroscopic outgrowth of tumor nodules does not occur, and therefore no direct evidence for an antitumor effect can be obtained. The preliminary experiment (table 1) and others performed more recently (Proctor JW, unpublished data) with a poorly immunogenic line of B16 melanoma in the C57BL/6J mouse strain of origin suggest, in fact, that an increased rate of cell clearance does correlate with a reduction in tumor outgrowth, in line with observations on immune animals (24,25). However, we consider that even these experiments are too unrepresentative of the clinical situation to be regarded as more than promising, and as a result, we have begun similar experiments on tumor-bearing mice.

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Antitumor Activity of Interferon Against Murine Osteogenic Sarcoma In Vitro and In Vivo [1]

Lowell A. Glasgow,* John L. Crane, Jr, Earl R. Kern, and Julius S. Youngner [2]

SUMMARY

Murine interferon inhibited the growth of a continuous line of osteogenic sarcoma cells in tissue culture. Inhibition of tumor cell growth was documented by decreased clone formation in liquid medium, decreased tumor cell counts in monolayer cultures, suppression of colony formation in semi-solid agar, and decreased uptake of ^{3}H-thymidine by the osteogenic sarcoma cells in culture. The capacity of anti-interferon antibody to block the tumor cell growth inhibitory activity of the interferon preparation suggested that interferon itself is the biologically active component of the interferon preparations. In vivo, a 7-day course of 30,000–60,000 units/day of type I interferon prepared in cell cultures either completely inhibited or delayed the appearance of tumors in experimental animals inoculated with osteogenic sarcoma cells by the sc route. The therapeutic efficacy of a preparation of murine sera containing type II interferon as well as other lymphokine activity was compared with the type I interferon preparation. Animals treated with 600 units of type II interferon were protected against tumor development as effectively as with 60,000 units/day of type I.

[Cancer Treat Rep 62:1881–1888, 1978]

Osteogenic sarcoma (OGS) is a common primary tumor of the bone in man. Although the prognosis has improved in recent years, the outcome of therapy has remained less than optimal. The studies to be reported in this paper were stimulated by the report by Strander and co-workers (1) that incorporation of human interferon into the therapeutic regimen of patients with OGS appeared to improve their outcome. Although these results are encouraging, the difficulty in defining the efficacy of one mode of therapy in complex human trials suggests that an experimental animal model should be developed which would permit evaluation of the tumor inhibitory activity of interferon under more controlled conditions.

In our laboratory we have developed a murine OGS cell line which was derived from a ^{239}Pu-induced tumor in order to provide an experimental model for evaluation of the effect of interferon on OGS (2). The establishment of this continuous line of OGS cells has provided an in vitro system for determination of the sensitivity of these cells to murine interferon which in turn permits a comparison with the sensitivity of human OGS cell lines to human interferon reported by Strander and Einhorn (3). The purpose of this study was to determine the effect of mouse interferon on the growth of murine OGS cells in cell culture as background for the evaluation of the effect of interferon treatment on the development of tumors in mice inoculated with OGS cells.

MATERIALS AND METHODS

Animals.—Six- to 8-week-old C57BL/6J female mice (Jackson Laboratories, Bar Harbor, Me) were used in all experiments. Animals were housed in an

[1] Supported in part by Public Health Service grant AI-10217 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health, Education, and Welfare.

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environment with 12-hour light and dark cycles, six animals per cage, with food and water ad libitum.

Tumor cells.—A continuous line of OGS cells was established from a ^{239}Pu-induced primary tumor in C57BL/6 mice (provided by Dr. Glenn Taylor, Division of Radiobiology, University of Utah, Salt Lake City). The cells were maintained and propagated in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum; glutamine, 100 μg/ml; penicillin, 100 units/ml; and streptomycin, 50 μg/ml in a 5% CO_2 atmosphere at 37°C. The OGS cells have remained tumorigenic when injected by the sc or ip route into C57BL/6J mice after 50 passages in vitro. In all experiments, mice were inoculated sc with 1×10^5 OGS cells/mouse in 0.1-ml volume. This concentration of cells resulted in the development of tumors in 70%–100% of the animals.

Interferon preparations.—The type I interferon preparation used was induced in C-243-3 mouse cells by Newcastle disease virus (Bionetics Laboratories, Kensington, Md). The stock preparation was received as two different lots and stored at –70°C until use. The antiviral activity of these type I interferon preparations at the beginning of the experiments was determined to be 300,000 and 160,000 units/ml by the 50% plaque-reduction assay in mouse L-929 cells (4). Subsequent antiviral assay of the two lots by the same method approximately 1 year later indicated a titer of 114,720 and 72,920 units/ml in L-929 cells and 60,770 and 18,320 units/ml in OGS cells. Titration of an internal laboratory standard, run simultaneously with each assay, was compared to the National Institutes of Health Murine Reference Standard (G002-904-511; accepted titer of 6500 units). The results of ten determinations indicated that the reference standard had a mean titer of 6850 ± 2675 units in our assay system. The initial titer of 300,000 and 160,000 units/ml for the two lots of interferon was used to determine the number of units per dose used in treatment schedules for each experiment. The type II interferon was a mouse sera preparation which had antiviral activity of 8000 units/ml. Both the type II interferon and serum control were prepared as previously described (5). Interferon antibody was received from the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases and was provided by Dr. K. Paucker (Medical College of Pennsylvania, Philadelphia). It was prepared in white rabbits immunized with L-cell interferon mixed with Freund's adjuvant as described by Ogburn and co-workers (6).

Tumor cell injection.—Tumor cells suspended in MEM at 1×10^6 viable cells/ml (determined by trypan blue exclusion) were injected sc (0.1-ml volume) into the right rear flank of shaved mice. The injection sight was marked to facilitate subsequent injection of interferon or control preparations.

RESULTS

In our initial experiments, OGS cells were seeded into 24 well plastic plates and grown for 3–7 days in the presence of 30,000–3 units of interferon. The striking inhibition of OGS cell growth is illustrated in figure 1. Almost complete inhibition was observed in the presence of 30,000 and 3000 units while decreasing degrees of inhibition were observed at 300, 30, and 3 units of interferon. To further document this growth inhibitory activity of interferon, the OGS cells present in the wells were trypsinized and counted after incubation for 72 hours in the presence of interferon. The results are presented in figure 2. The decreased number of OGS cells present in wells exposed to 30,000–300 units of interferon in comparison with the absence of inhibition of mouse embryo fibroblast cells supports the evidence that interferon has a selective capacity to inhibit the growth of malignant cells.

One characteristic of tumor cells is their capacity to form colonies in semi-solid agar. To further document the effect of interferon on the growth of OGS cells, 2×10^4 cells were incubated in 0.25% agarose containing serial dilutions of interferon. Colony development was observed for 3 weeks. The photomicrographs in figure 3 illustrate the inhibition of colony formation in the cultures containing interferon in comparison with control cultures. This inhibition was evidenced by a decrease in both size and number of colonies in the presence of ≥ 300 units of interferon.

In order to further delineate the effects of interferon on the growth of OGS cells, the rate of uptake of ^3H-thymidine was determined in cultures of OGS cells exposed to serial dilutions of interferon. In these experiments, approximately 2×10^4 OGS cells were incubated in 6-mm wells to which 1 microcurie/well of ^3H-thymidine was added for a 4-hour pulse at 24 or 72 hours after incubation. The total number of counts taken up by the cells in the presence of serial tenfold dilutions of interferon is presented in figure 4. At both 24 and 72 hours, 30,000–300 units of interferon reduced the uptake of ^3H-thymidine in the OGS cells. Less inhibition was observed at 30 units and inhibition was lost in cultures incubated with only 3 units of interferon.

During the past several years, there has been debate about whether the antitumor effect of interferon is due to interferon itself or to a contaminant in the interferon preparation. The next series of experiments were designed to determine whether

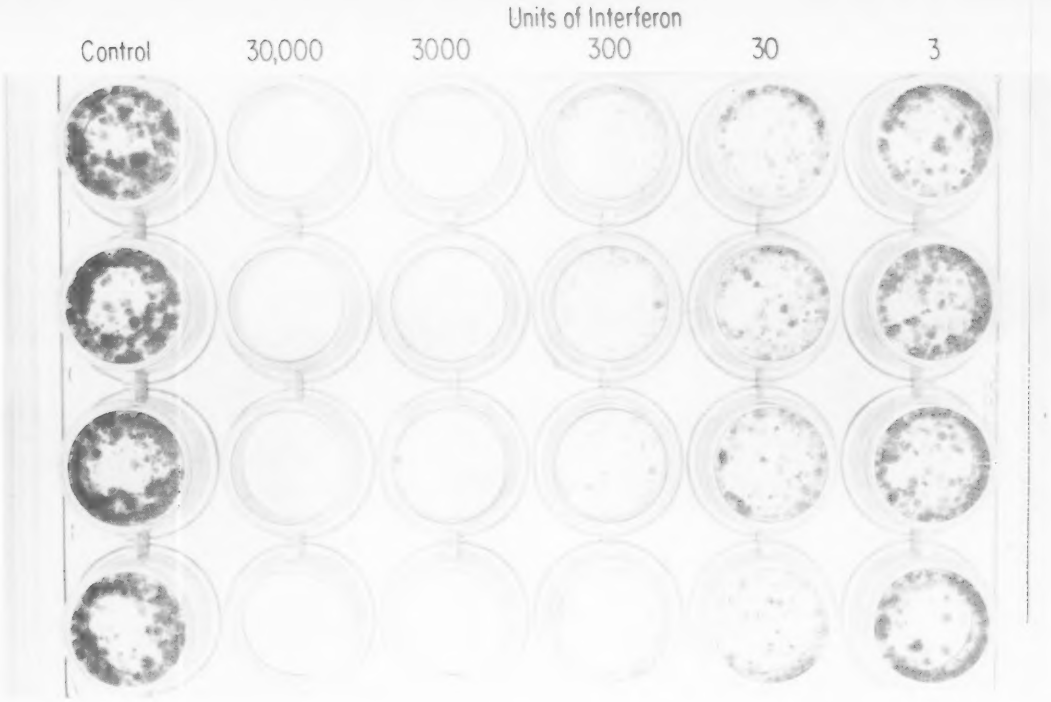

FIGURE 1.—Inhibition of murine OGS cells in the presence of serial dilutions of murine interferon. Plates were photographed after 9 days of incubation (reproduced *from* ref 2).

anti-interferon antibody would prevent the inhibitory activity of our interferon preparations against OGS cells. Serial dilutions of anti-interferon antibody were incubated with OGS cells grown in the presence of either 3000 or 300 units of interferon. After 10 days of incubation, the medium was removed from the plates and the colonies of cells were stained. The photograph of one representative plate is presented in figure 5. It is evident that the cell inhibitory activity of 3000 units of interferon was reversed at a 1:200 dilution of antibody, but not with higher dilution. In contrast, dilutions of antibody up to 1:1600 clearly suppressed the cell inhibitory activity of 300 units of interferon while at 1:3200 and 1:6400 dilutions of the antibody decreased activity was seen. These results support the hypothesis that interferon is the active cell inhibitory factor in our preparations.

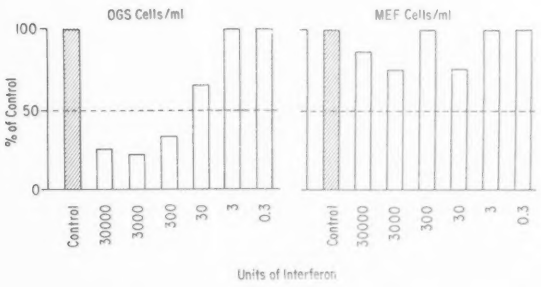

FIGURE 2.—Inhibition of growth of murine OGS cells compared with mouse embryo fibroblast cells in tissue culture in the presence of serial dilutions of murine interferon (30,000–0.3 units). Cell counts were made after 72 hrs of exposure to interferon. Mean cell counts (total of 3 wells) are expressed as % of control (reproduced *from* ref 2).

These in vitro studies confirm that interferon has the capacity to inhibit tumor cell growth in vitro and that this activity could be demonstrated in a striking fashion against a murine OGS cell line. In the next series of experiments, our studies were extended to determine the effect of interferon treatment on the development of tumors in mice inoculated with OGS cells. Inoculation of 1×10^5 OGS cells sc in the flank resulted in the development of tumors over a 2–4-week period. In an initial experiment, therapy was started immediately after the inoculation of OGS cells with 16,000 units of interferon administered twice daily by sc injections at

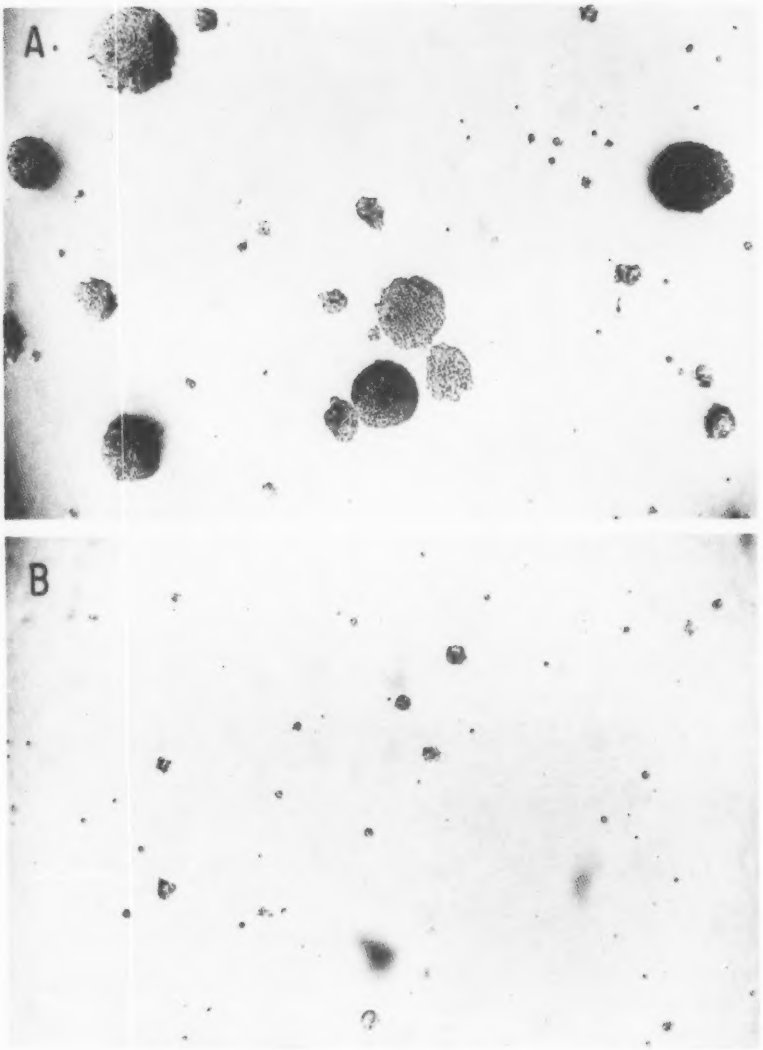

FIGURE 3.—Inhibition of OGS cell colony formation in soft agar by incorporation of 3000 units of interferon in the agar (B) compared with a control culture (A). Photographs were taken after 14 days of incubation (× 31) (reproduced *from* ref 2).

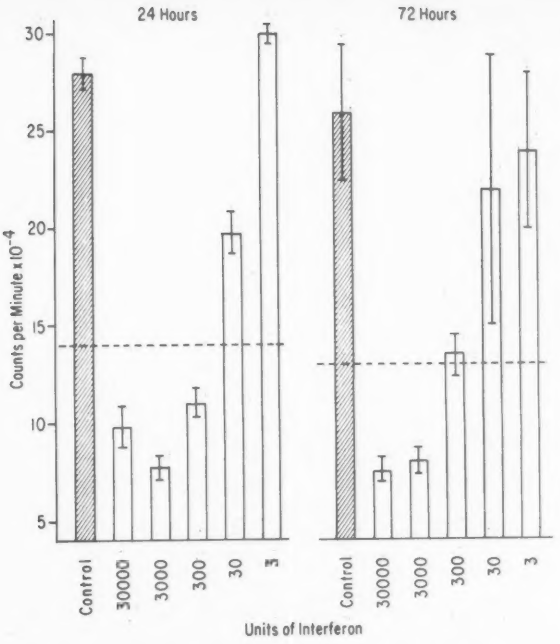

FIGURE 4.—Inhibition of uptake of ^{3}H-thymidine in OGS cells after incubation in the presence of interferon for 24 and 72 hrs (reproduced *from* ref 2).

the approximate site of tumor cell inoculation. Although tumors eventually developed in all mice, the appearance of palpable tumors in the interferon-treated mice was delayed for > 20 days after the initial development of palpable tumors in the control group. The mean day of death in the interferon-treated group was 24 days later than that for the control group. Although these differences were not statistically different because of the small numbers of animals, the results suggested that interferon might have efficacy in vivo against this transplantable OGS. In a second experiment, a similar protocol was followed except that groups of five to ten animals were treated with 30,000 units of interferon per injection with treatment beginning immediately after inoculation of the OGS cells and continued on a twice daily basis for 7 days. The results from this experiment are summarized in figure 6. Animals receiving interferon were protected against tumor development as evidenced by a delay of 40 days in the initial appearance of tumors in comparison with control animals receiving injections of tissue culture medium. Only 80% of the interferon-treated animals developed tumors and eventually died compared with 100% of control animals.

These data suggest that interferon is effective in inhibiting the development of a transplantable OGS in vivo. Salvin and co-workers (7) have presented evidence that murine sera containing relatively small amounts of type II interferon, as well as other lymphokine activity, are effective in inhibiting a transplantable tumor in experimental animals. In the next series of experiments, animals were treated with a murine sera preparation containing 300 units of type II interferon administered sc twice daily for 7 days at the approximate site of OGS cell inoculation. Other groups of experimental animals received either 300 or 30,000 units of type I interferon according to the same therapeutic regimen or a control preparation of murine sera. The results are presented in figure 7. The type II interferon-containing serum appears to be highly effective in inhibiting tumor development. No sc tumors developed after an observation period of 70 days in animals receiving this interferon preparation. The administration of 60,000 units of type I interferon was also effective in preventing tumor development while 600 units of type I had no effect. All control animals developed tumors within 16 days as did all animals receiving 600 units of type I interferon. In contrast, only 10% of the animals treated with 60,000 units/day of type I interferon and 20% of the animals treated with the murine sera containing 600 units of type II interferon eventually developed tumors. These data suggest that both interferon preparations are effective but that the serum preparation containing type II interferon is strikingly more effective than an equal number of antiviral units of type I interferon under the conditions of these experiments. These results confirm the capacity of interferon to inhibit the growth of tumor cells both in vitro and in vivo.

DISCUSSION

It is becoming apparent that interferon has a broad range of biologic activities in addition to its capacity to inhibit virus replication (8,9). The present studies were stimulated by the evidence presented by Strander and co-workers (1) who initially presented evidence that treatment of patients diagnosed as having OGS with long-term administration of human leukocyte interferon as part of their therapeutic regimen resulted in an increase in the time for metastases to develop. The availability of other modes of therapy and the current limitations on human experimentation make it difficult to carry out the clinical experiments necessary to define the effect of interferon in the treatment of this form of malignancy in man. The development of an OGS cell

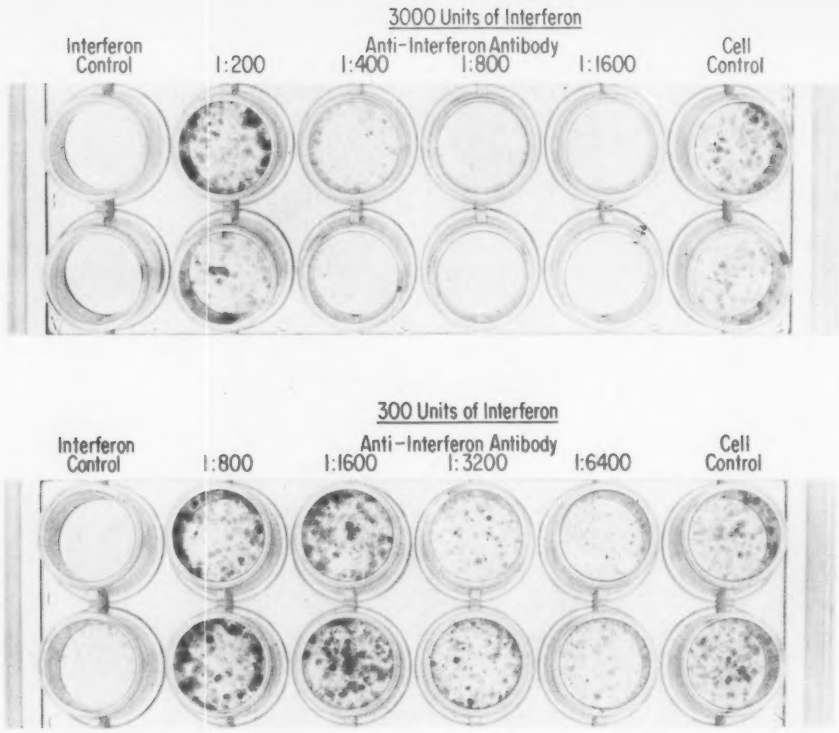

FIGURE 5.—Prevention of tumor cell inhibitory activity of 3000 (top) and 300 (bottom) units of interferon by serial dilutions of anti-interferon antibody (reproduced *from* ref 2).

line which may be transplanted into experimental animals provides a model for the evaluation of potential chemotherapeutic modalities under more controlled conditions.

Our initial series of experiments in vitro demonstrated that the continuous cell line which we established from a ^{239}Pu-induced OGS is sensitive to the inhibitory activity of murine interferon. This antitumor activity of interferon was documented by its capacity to reduce tumor cell growth in culture, to decrease colony formation in soft agar, to suppress clone formation in liquid medium, and to inhibit ^{3}H-thymidine uptake by the OGS cells. These results parallel the tissue culture studies of Strander and Einhorn (3) who demonstrated that human leukocyte interferon inhibited the growth of human OGS cell lines.

Although conflicting evidence has been presented concerning the question of whether interferon has cell inhibitory activity or a contaminant in interferon preparations is the active factor, the weight of current evidence favors the interpretation that interferon has both antiviral and cell growth inhibitory activities. The experimental data presented here would support this concept. Anti-interferon antibody prepared by Dr. Paucker against his interferon preparations was highly active in preventing our interferon preparation from inhibiting the growth of OGS cells. It should be noted, however, that neither interferon preparation was purified and that a common contaminating substance could have been present in both interferon preparations.

The antitumor activity of interferon has previously been demonstrated in a number of animal models including malignancies that occur spontaneously (10) or were induced by irradiation (11), chemical carcinogen (12), Rauscher or Friend leukemia virus (13,14), Moloney sarcoma virus (15), or transplantable tumors (7,9,16,17). Although interferon was administered for a relatively short period of time in our experiments (7–9 days), 32,000–60,000 units/day of type I interferon effectively suppressed the transplantable OGS tumor.

It is interesting that relatively low doses of type II interferon (600 units) administered by a similar regimen were strikingly effective in inhibiting tu-

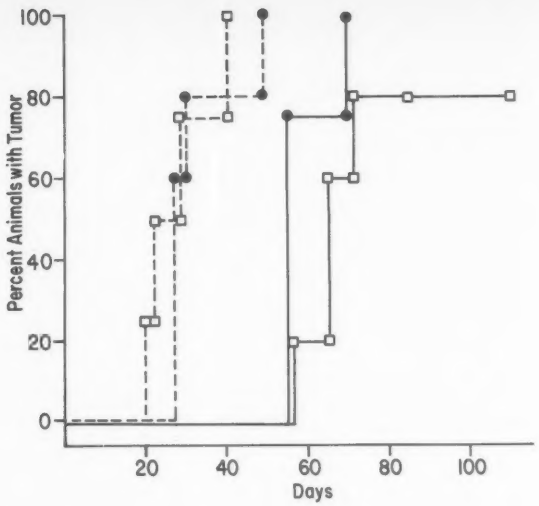

FIGURE 6.—Development of tumors in C57BL/6J mice after inoculation of 1×10^5 OGS cells. Experiment 1: control (●----●); 32,000 units of interferon/day (●——●). Experiment 2: control (□------□); 60,000 units of interferon/day (□——□). (Reproduced *from* Glasgow LA, Crane JL, Jr, and Kern ER. J Natl Cancer Inst, vol 61, pp 871–874.)

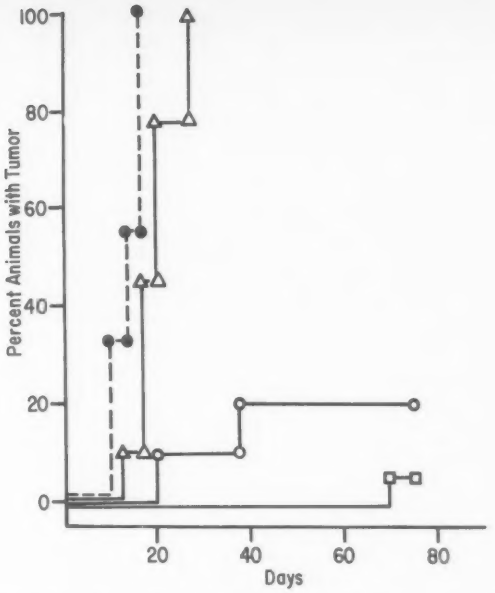

FIGURE 7.—Comparison of antitumor activity of type I and II interferon in C57BL/6J mice inoculated with 1×10^5 OGS cells. Normal serum (●----●); 600 units of type I interferon/day (△——△); 60,000 units of type I interferon/day (□——□); 600 units of type II interferon/day (○——○). (Reproduced *from* Glasgow LA, Crane JL, Jr, and Kern ER. J Natl Cancer Inst, vol 61, pp 871–874.)

mor development. These results confirm the work of Salvin and co-workers (7) who described the suppression of growth of a transplantable methylcholanthrene-induced tumor with the daily administration of 300 units of type II murine interferon. The reason for the greater activity of the murine serum preparations containing type II interferon has not been elucidated. The fact that these preparations also contain lymphokine activity, and more specifically, migration inhibition factor, suggest that activation of macrophages may be one factor in the enhanced antitumor activity of these preparations. This hypothesis is supported by evidence from our laboratory that activated macrophages from C57BL/6J mice are tumoricidal for the OGS cells (18) and by the previous work of Gresser et al (9) which has shown that host-mediated factors were a component of the enhanced resistance to development of tumors in experimental animals treated with interferon. They further demonstrated that tumor cells were phagocytized by macrophages from interferon-treated animals, but not controls.

In summary, the evidence presented in this report demonstrates that murine interferon effectively inhibits the replication of OGS cells in vitro. The sensitivity of this tumor to the growth inhibitory activity of interferon was extended to experimental animals where both type I and II interferon were shown to inhibit the development of transplantable OGS tumors. Since both murine and human OGS cells are sensitive to interferon in tissue culture, the present data suggest that our murine OGS system will provide a model for evaluation of treatment of OGS in man. It is hoped that this model will provide an opportunity to better define optimal treatment schedules and to determine whether established tumors can be treated and if interferon can prevent the development of metastatic lesions. Finally, the striking differences in the activity between type I and II interferon suggest that the mechanism of action of these two preparations should be defined to determine whether greater activity may be achieved through the use of immune, type II interferon.

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Cytotoxic Activity of Interferon-Treated Macrophages Studied by Various Inhibitors

Richard M. Schultz,* Nicholas A. Pavlidis, William A. Stylos, and Michael A. Chirigos[1]

SUMMARY

The ability of interferon-treated macrophages to kill or inhibit the growth of tumor cells is markedly influenced by the local environment. The macrophage cytotoxic effector system is regulated by serum and other environmental factors that suppress tumor killing. Prostaglandins E_1 and E_2, but not $F_{2\alpha}$, reversibly inhibited the tumoricidal state of interferon-treated macrophages. Hydrocortisone was similarly active at suppressing macrophage function. Such nonimmunologically derived factors could prevent the final triggering step for macrophage-mediated tumor killing in the local environment of the tumor and may be important in the pathogenesis of progressive tumor growth.

[Cancer Treat Rep 62:1889–1892, 1978]

Macrophage tumoricidal function appears to be influenced by a number of opposing signals in the local environment of the cell. Activating stimuli, such as interferon liberated from fibroblast cultures (1,2), numerous polyanionic interferon inducers (3), bacterial endotoxins (4,5), and lymphokine released from antigen- or mitogen-stimulated lymphocytes (6) render macrophages selectively cytotoxic for tumor cells. However, conflicting stimuli, such as proteases (7), tumor secretory products (8), high molecular weight lipids (7), and hydrocortisone (9), may compromise the clinical usefulness of macrophage-activating agents.

Several investigators have demonstrated that the ability of immunopotentiators to enhance host resistance to cancer correlates with their capacity to render macrophages nonspecifically tumoricidal (10–12). Both the macrophage functional activity and concentration at the primary and disseminated tumor sites appear to be intimately related to the clinical course of neoplastic disease. The present communication concerns itself with the effects of fibroblast interferon on macrophages and demonstrates some pharmacologic agents that are capable of abrogating macrophage-mediated cytotoxicity. Sufficient concentrations of these opposing factors in the microenvironment of the tumor could be important contributing factors in the tumor's progression.

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MATERIALS AND METHODS

Mice

Male $CD2F_1$ mice, 6–8 weeks old, were obtained from the Mammalian Genetics and Animal Production Section, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. The mice were housed in plastic cages and fed Purina laboratory chow and tap water ad libitum.

Drugs

Partially purified mouse L-cell interferon (specific activity, 2×10^7 units/mg protein) was purchased from Dr. K. Paucker, Medical College of Pennsylvania, Philadelphia. Interferon was purified by affinity chromatography on anti-interferon globulin-sepharose (13). Prostaglandins E_1, E_2, and $F_{2\alpha}$ (PGE_1, PGE_2 and $PGF_{2\alpha}$) were generously furnished by Dr. John Pike of The Upjohn Co, Kalamazoo, Mich. Hydrocortisone and indomethacin were obtained from Sigma Chemical Co, St. Louis, Mo.

Cell Cultures

Leukemia cell cultures.—MBL-2 lymphoblastic leukemia cells were maintained as suspension cultures in RPMI-1640 medium supplemented with 20% heat-inactivated (56°C for 30 minutes) fetal calf serum (FCS), 100 μg/ml gentamicin solution, 0.075% $NaHCO_3$, and 10 mM Hepes buffer (RPMI-FCS). Viability of the cells was determined by the trypan-blue exclusion test.

Peritoneal macrophages.—Noninduced peritoneal macrophages were harvested from mice as previously described (14) and washed in RPMI-1640 medium, and approximately 4×10^5 macrophages were seeded into 16-mm wells on tissue culture Cluster[24] plates (Costar, Cambridge, Mass) in 1.0 ml of RPMI-FCS. The cultures were incubated for 90 minutes, and macrophage monolayers were washed thoroughly with jets of medium before use in experiments.

Macrophage Activation Assay

Our technique for measuring the ability of interferon to produce growth inhibitory macrophages in vitro has previously been described (15). Monolayers of macrophages in 16-mm wells were treated with partially purified interferon (lot No. 1835) at a final concentration of 1000 units/ml culture medium. Agents tested for inhibitory activity on macrophage function were added in 100-μl aliquots to the macrophage cultures either for the duration of exposure to interferon (16 hours at 37°C) or for a 2-hour period of incubation following the 16-hour interferon treatment. Following the incubation period, the macrophage cultures were washed three times with medium to remove interferon and/or inhibitor, and the macrophages were overlaid with 4×10^4 MBL-2 lymphoblastic leukemia cells contained in 2 ml of RPMI-FCS. All cultures were again incubated at 37°C in an atmosphere of 5% CO_2 in air, and viable leukemia cells were counted after 48 hours with a hemocytometer. The ratio of macrophages to target cells was approximately 10:1 at the beginning of each experiment. The percent growth inhibition of MBL-2 cells due to macrophage-interferon interaction was calculated by comparison to MBL-2 cells grown in the presence of normal resting macrophages alone.

RESULTS

Activation of Macrophages by Interferon in Vitro

When macrophages were exposed to partially purified interferon at 1000 units/ml for 16 hours and washed prior to addition of MBL-2 target cells, the macrophages suppressed the leukemia cell growth at 48 hours by a mean ± standard error from eight separate experiments of 67.1% ± 3.4%. Resident macrophages from untreated mice did not influence MBL-2 proliferation. Macrophage tumoricidal capacity was accompanied by increased spreading of macrophages on plastic and by increased adherence of tumor cells on macrophage membranes. The cytotoxicity of interferon-treated macrophages has previously been characterized to require cell-to-cell contact between macrophage and target cell (1,2).

Effect of Adult Mouse Sera on Macrophage Tumoricidal Function

Peritoneal macrophages treated with interferon kill tumor cells when the culture medium contains FCS (1,2). We examined the effect of adult mouse sera on activated macrophage tumor killing. Pools of serum were obtained from 8-week-old $CD2F_1$ and C57BL/6 mice and were heat-inactivated at 56°C for 30 minutes prior to testing. When normal adult mouse sera were added to macrophage cultures simultaneously with the interferon stimulus, the ability of interferon to activate macrophages was abrogated (fig 1). $CD2F_1$ sera were still active at 1.2% and C57BL/6 sera were still active at 2.5% at inhibiting cytotoxic function. Thus, serum factors exist which may compromise the antitumor activity of interferon-treated macrophages.

Effect of Prostaglandins on Macrophage Tumoricidal Function

When PGE_1 or PGE_2 was added to macrophage cultures either simultaneously with interferon or for a 2-hour pulse immediately following interferon treatment, the interferon induction of tumoricidal macrophages was inhibited (table 1), although macrophages were observed to be activated as defined by morphologic criteria and remained fully viable. Both PGE_1 and PGE_2 showed similar kinetics of inhibiting macrophage function. In contrast, $PGF_{2\alpha}$ was ineffective at all concentrations tested. The prostaglandins or the prostaglandin synthetase inhibitor, indomethacin, was without effect on resting macrophages. Since PGE_1 and PGE_2 could inhibit expression of tumoricidal function after the macrophages had reached their cytotoxic potential, the modulation observed probably involved events already initiated by the interferon stimulus.

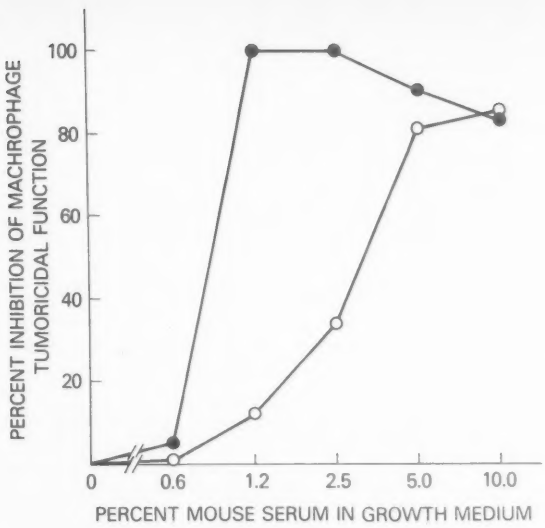

FIGURE 1.—Effects of adult mouse sera on tumoricidal function of interferon-treated macrophages. $CD2F_1$ (●) and C57BL/6 (○) sera were added simultaneously with interferon (1000 units/ml) to monolayers of resting $CD2F_1$ macrophages for 16 hrs at 37°C. The cultures were then washed, and MBL-2 leukemia target cells were added. Cytotoxicity was determined after a 48-hr coincubation with target cells at 37°C. Each point represents the mean obtained from 3 determinations.

Effect of Hydrocortisone on Macrophage Tumoricidal Function

When macrophages were pulsed for 2 hours with hydrocortisone after interferon treatment, their cytotoxic activity was inhibited (table 2). Strong inhibitory effect was seen with doses as low as 1×10^{-7} M. The ability of hydrocortisone to inhibit macrophage tumoricidal function has previously been suggested to result from macrophage membrane stabilization and subsequent inhibition of transfer of activated macrophage lysosomes into the target cell (9).

DISCUSSION

Macrophage tumoricidal function appears to be a relative capacity that is markedly influenced by the local environment. This communication shows that the macrophage cytotoxic effector system is modulated by serum and other environmental factors including prostaglandins of the E series and hydrocortisone that can potently suppress tumor killing.

Prostaglandins of the E series have previously been shown to exert a number of inhibitory effects on macrophages besides cytotoxicity, including decreased random locomotion (16), and lessened production of plasminogen activator (17) and colony-stimulating factors for macrophage stem cells (18).

TABLE 1.—Effect of prostaglandins on macrophage tumoricidal function

Inhibitor	Concentration of inhibitor (M)	Incubation period*		Inhibition of tumoricidal function (%)
		Simultaneous with interferon	After interferon	
PGE_1	10^{-5}	+	–	94
	10^{-6}	+	–	63
	10^{-7}	+	–	23
	10^{-8}	+	–	0
	10^{-5}	–	+	57
PGE_2	10^{-5}	+	–	93
	10^{-6}	+	–	57
	10^{-7}	+	–	38
	10^{-8}	+	–	18
	10^{-5}	–	+	72
$PGF_{2\alpha}$	10^{-5}	+	–	10
	10^{-6}	+	–	10
	10^{-7}	+	–	13
	10^{-8}	+	–	8
	10^{-5}	–	+	0

*Prostaglandins were added either simultaneously with interferon or for a 2-hr pulse after interferon treatment. Macrophage monolayers were then washed several times prior to the addition of target cells, and cytotoxic function was assessed after 48 hrs.

TABLE 2.—Effect of hydrocortisone on macrophage tumoricidal function*

Concentration of hydrocortisone (M)	Inhibition of cytotoxic activity (%)
10^{-6}	86
10^{-7}	61
10^{-8}	7
10^{-9}	−6

*Macrophages were exposed to hydrocortisone for 2 hrs immediately following interferon treatment. Macrophage monolayers were washed several times prior to the addition of MBL-2 target cells, and cytotoxic function was assessed after a 48-hr incubation at 37°C.

The process of macrophage activation appears to be self-limiting, since activated macrophages release high levels of PGE_2 (19). Such prostaglandins could act in negative feedback inhibition to limit cell activities. Preliminary evidence suggests that the ability of PGEs to inhibit macrophage cytotoxic function is related to elevated levels of intracellular cyclic 3′5′ adenosine monophosphate. In addition, cells transformed by viruses or chemicals in vitro have been shown to produce several-fold more prostaglandins than their normal counterparts (20). These tumor cell products could subvert local macrophages and allow the tumor to progress in the presence of these potentially tumoricidal cells.

In conclusion, a more comprehensive knowledge of the stimuli and conditions under which the cytotoxic activity of macrophages is maintained in vivo may clarify the role of the activated macrophage in tumor immunity. In the past, much emphasis has been placed on factors that enhance the functional activity of the macrophage system. It appears that there are opposing factors that may subvert the functional activity of the activated macrophage. Since the effects of the inhibitors are readily reversible (unpublished observation), the effects are often lost when cells are placed in vitro. It is possible to envision that the future of immunotherapy may not only be in using agents that produce macrophage tumor killing, but also in using agents that will block the production or release of host factors that abrogate macrophage effector function in the local tumor environment.

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Role of Interferon in Augmentation of Natural and Antibody-Dependent Cell-Mediated Cytotoxicity

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SUMMARY

Natural killer (NK) activity in rats and NK and antibody-dependent cell-mediated cytotoxicity in mice have been shown to be rapidly augmented by in vivo treatment with a variety of viruses and immune adjuvants. Poly I:C and other interferon inducers also caused boosting in NK-cell activity, with peak levels occurring at about the time of peak interferon levels. Crude and purified virus-induced interferon itself had strong boosting effects in mice, both when inoculated in vivo and when incubated briefly with normal spleen cells. The in vitro activation by interferon or by poly I:C was completely abrogated by anti-interferon. Interferon also appears to play a central role in augmentation of human NK-cell activity. Inoculation of normal volunteers with influenza virus or of a patient with poly I:C produced rapid boosting. In vitro incubation of peripheral blood mononuclear cells with various interferon preparations for 1 hour or overnight usually produced an appreciable increase in lytic activity.

[Cancer Treat Rep 62:1893–1896, 1978]

In the past several years, it has become increasingly apparent that normal subjects, as well as tumor-bearing patients, may have considerable levels of cytotoxic reactivity against tumor cells. In both rodents and man, this natural cell-mediated cytotoxicity has been found to be mediated by a particular subpopulation of lymphocytes, which have been designated natural killer (NK) cells (1). In recent studies in our laboratory, we have been impressed by many similarities between the NK and the effector (K) cells mediating antibody-dependent cell-mediated cytotoxicity against tumor target cells. We have come to the conclusion that NK and K cells belong to the same subpopulation of lymphocytes. These cells do not appear to be thymus-dependent, since high levels of NK activity are found in nude mice, but NK cells may be pre-T cells, since in the mouse they have a low density of theta antigen (2) and in man they have low affinity receptors for sheep erythrocytes (3). NK cells may play an important role in the in vivo resistance against tumor growth and in immunosurveillance.

In mice and rats, NK-cell activity is usually first detectable at 3–4 weeks of age, reaches a peak at 5–8 weeks, and then declines to low or undetectable levels after 10–12 weeks. In man, similar levels of NK activity have been seen in patients over a wide age range and some activity has even been detected in cord blood lymphocytes. It has been of considerable interest to determine the factors responsible for induction of NK activity and for regulation of the levels of this K-cell function. Some insight into this issue was first provided by the observation (4) that NK activity in mice could be strongly and rapidly augmented in vivo by inoculation with a variety of materials, including various murine viruses and the bacterial adjuvants, bacillus Calmette-Guérin and *Corynebacterium parvum* (table 1). These treatments had similar effects on levels of K-cell activity.[3] Of particular interest, we have been able to

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[3]Santoni A, Herberman RB, and Holden HT. Correlation between natural and antibody-dependent cell-mediated cytotoxicity against tumor targets in the mouse. I. Distribution of the reactivity. J Natl Cancer Inst. In press, 1979.

TABLE 1.—Boosting of spleen cell NK activity in nude mice

Challenge material	% cytotoxicity*	
	No challenge	Challenge
Murine sarcoma virus	11.6	35.7
Lymphocytic choriomeningitis virus	10.2	42.0
Mouse hepatitis virus	10.2	42.4
Bacillus Calmette-Guérin	13.7	20.3
C. parvum	13.7	23.7

* Attacker:target cell ratio of 50:1 against RL♂1.

TABLE 2.—In vivo effects of interferon inducers on NK activity of C57BL/6N spleen cells

Treatment	% cytotoxicity* at—		
	3 hrs	16 hrs	3 days
None	2.5	2.8	2.9
Balanced salt solution	3.1	0.9	3.2
Pyran (25 mg/kg)		6.7	12.6
Lipopolysaccharide (100 μg)	16.8	10.3	
Poly I:C (100 μg)	9.3	12.8	3.7
Poly I:C (10 μg)		9.2	3.5
Poly I:C (10 μg) × 3 days		26.0	

* Effector:target cell ratio of 200:1 against RL♂1.

induce strong NK- and K-cell activities in older mice at a time when spontaneous levels were very low or undetectable. Studies of the kinetics of this response showed a characteristic peak in the spleen at 3 days and then a decline to baseline levels by 5–7 days.

The ability to boost NK- and K-cell activities by a variety of agents caused us to seek a common factor which could explain all of these effects. Interferon appeared to be a good candidate, since the active viruses and also *C. parvum* have been shown to induce interferon with kinetics similar to those observed for generation of NK activity. This possibility of a role of interferon was further supported by studies of Oehler et al (5), indicating that poly I:C, as well as *C. parvum* and some viruses, could induce augmented NK activity in rats. We therefore initiated a detailed series of experiments in mice to determine the role of interferon in boosting NK.[4] Poly I:C and a variety of other interferon-inducing agents were shown to be able to induce considerable increases in cytotoxic reactivity, and the peak responses occurred around the time of previously described rises in interferon levels (table 2).

[4]Djeu JY, Heinbaugh JA, Holden HT, et al. Augmentation of mouse natural killer cell activity by interferon and interferon inducers. J Immunol. In press, 1979.

TABLE 3.—In vivo effects of interferon on NK activity of C57BL/6N spleen cells

Treatment	% cytotoxicity* at—		
	3 hrs	16 hrs	42 hrs
None	3.0	3.0	2.8
Balanced salt solution	3.7	3.7	0.9
Crude interferon			
2×10^6 units	13.2	8.9	3.9
2×10^5 units	3.9	4.2	3.0
Purified interferon			
1×10^4 units	14.7		

* Effector:target cell ratio of 200:1 against RL♂1.

These findings encouraged us to directly examine the in vivo effects of interferon itself. Administration of crude or affinity-column purified virus-induced interferon to mice induced an increase in NK activity that could be detected within 3 hours (table 3). Gidlund et al[5] have also shown that in vivo administration of interferon inducers and interferon could induce augmentation of NK activity and that simultaneous in vivo administration of anti-interferon could efficiently block the effects of the interferon inducers and could partially block the effects of *C. parvum*.

To analyze the processes involved in the boosting of mouse NK activity in more detail, we have set up an in vitro system for examining the effects of interferon inducers or interferon. Although mouse NK activity is more labile at 37°C than cytotoxicity mediated by immune, mature T cells, we have been able to at least partially overcome this by manipulation of the culture conditions. Incubation of spleen cells from older mice with poly I:C for 2 hours, followed by overnight incubation, resulted in augmented levels of NK activity (table 4). Interferon itself was also active and the effects were more rapid, with a detectable increase in NK activity within 45 minutes after exposure. The boosting of NK activity by poly I:C in vivo and in vitro has been dependent on macrophages, which appear to be the main cells induced to make interferon.[6] In contrast, direct activation of NK cells by interferon has not been dependent on macrophages. To further document the role of interferon in this augmentation, we performed experiments with an antiserum to purified mouse interferon, kindly provided by Dr.

[5]Gidlund M, Örn A, Wigzell H, et al. Enhanced NK cell activity in mice injected with interferon and interferon inducers. *Nature* 273:759–761, 1978.

[6]Djeu JY, Heinbaugh JA, Holden HT, et al. Role of macrophages in the augmentation of mouse natural killer cell activity by an interferon inducer and by interferon. J Immunol. In press, 1979.

TABLE 4.—In vitro effects of poly I:C and interferon on NK of C57BL/6N spleen cells

Treatment	% cytotoxicity*
None	5.0
Poly I:C	
100 μg, 2 hrs	18.3
100 μg, 18 hrs	28.4
1 μg, 18 hrs	10.6
Interferon	
Crude, 10^4 units, 2 hrs	17.1
Crude, 10^3 units, 2 hrs	8.3
Purified, 10^3 units, 45 mins	12.5
Purified, 10^2 units, 45 mins	9.1

* Effector:target cell ratio of 200:1 against RL♂1.

TABLE 5.—Effect of anti-interferon on in vitro boosting of NK by poly I:C and interferon

Treatment of CBA/J spleen cells	% cytotoxicity*
None	15.6
Interferon (2000 units)	33.4
+ anti-interferon	13.1
+ control globulin	30.8
None	4.6
Poly I:C (100 μg)	21.5
+ anti-interferon	1.6
+ control globulin	31.7

* Effector:target cell ratio of 200:1 against RL♂1.

Ion Gresser. When this antiserum was added to cultures of mouse spleen cells with either poly I:C or interferon, it was able to completely block the augmentation (table 5).

Since different mouse strains have a rather wide range in spontaneous NK levels, it was of interest to determine whether differences in responsiveness to interferon might be one of the factors responsible for this variation. As a preliminary attempt to analyze this, spleen cells from CBA/N mice, a strain with very high NK activity, and from C57BL/6N mice, a strain with intermediate levels of NK activity, were treated with interferon (table 6). The spleen cells were obtained from mice > 12 weeks of age, a time when the NK activity of untreated cells of both strains would be low. After incubation for 1 hour with interferon, much higher levels developed in the CBA/N spleen cells as compared to the C57BL/6N cells. In this experiment, we also tested spleen cells from C57BL/6N mice which had been inoculated 3 days earlier with lymphocytic choriomeningitis virus. Interferon had little boosting effect on these cells, which may have already been strongly stimulated by interferon in vivo.

TABLE 6.—In vitro effects of interferon on various mouse spleen cell populations

Treatment	% cytotoxicity*		
	CBA/N	C57BL/6N	C57BL/6N, 3 days after lymphocytic choriomeningitis virus
None	6.3	3.1	26.7
Balanced salt solution	5.5	2.8	21.2
Interferon			
2×10^4 units	21.2	9.6	31.1
2×10^3 units	17.7	6.7	24.8

* Effector:target cell ratio of 200:1 against RL♂1.

We have recently been performing analogous experiments in man, to determine if interferon also plays an important role in regulating human NK- and K-cell activities. We initially observed that normal subjects with intercurrent virus infections had transient, marked elevations in NK- and K-cell activities. The boosting effects of viruses were supported by studies of the effects of attenuated influenza virus and of swine influenza virus vaccine in normal subjects. Increased NK- and K-cell activities were noted 2–3 days after administration. We very recently had the opportunity to study the effects of poly I:C-poly lysine complex on one patient of Dr. King Engel, with amyotrophic lateral sclerosis. Increased NK- and K-cell activities were observed 2 days after inoculation, with rapid return to baseline levels thereafter.

In vitro studies with human peripheral blood mononuclear cells have indicated that the addition of interferon prepared from either human fibroblasts, leukocytes, or lymphoblastoid cell lines caused substantial increases in NK- and K-cell activities. However, the optimal time of exposure has varied among individual donors (table 7). In about half of the experiments, preincubation with interferon for 1 hour resulted in an appreciable boosting, with a sixfold increase in NK activity in one case. Overnight incubation of lymphocytes with interferon has resulted in somewhat more consistent augmentation of NK- and K-cell activities. These variations in the incidence and magnitude of responsiveness of different subjects may be analogous to the experiments in mice, in which sensitivity to interferon effects varied between strains and was influenced by previous in vivo exposure to activating stimuli. As confirmation of the role of interferon

TABLE 7.—In vitro effects of interferon on NK- and K-cell activities of peripheral blood lymphocytes of normal donors

Treatment time	Natural cytotoxicity			Antibody-dependent cell-mediated cytotoxicity		
	Median	Range	No. of experiments > 150% of control/total experiments	Median	Range	No. of experiments > 150% of control/total experiments
1 hr	160*	60–1626	10/20	130	90–460	3/9
18–20 hrs	195	130–290	4/6	280	94–490	5/6

* Activity after treatment as % of activity (calculated as lytic units/10^7 cells) in untreated control lymphocytes.

in mediating the in vitro activation, we have found that the addition of anti-interferon inhibited these effects. These data substantiate the recent suggestion by Trinchieri (6) of an enhancing effect of interferon on NK-cell activity. However, in contrast to his data, we have observed similar effects on both K-cell and NK-cell activity.

The findings described here indicate that interferon plays a central role in activating rodent and human NK and K cells. This rapid and strong potentiation of cytotoxic K-cell activities by interferon has important implications for in vivo resistance against tumor growth. These effects need to be seriously considered as possible mechanisms for the antitumor effects of a variety of immune adjuvants, as well as those of interferon itself. The monitoring of the effects of in vivo therapy on levels of NK- and K-cell activities may aid in the development of optimal dosages and schedules for immunotherapy for cancer.

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Human Interferon and Its Inducers: Clinical Program Overview at Roswell Park Memorial Institute [1]

William A. Carter* and Julius S. Horoszewicz [2]

SUMMARY

An overview of the clinical interferon program at Roswell Park Memorial Institute is presented. Purified fibroblast interferon and a novel inducer of human interferon [$rI_n \cdot r(C_{12},U)_n$] are being evaluated for possible antiviral, antiproliferative, and immunomodulatory activities in patients with cancer.

[Cancer Treat Rep 62:1897–1898, 1978]

Human fibroblast interferon (HFIF) is a glycoprotein which possesses antiviral, antiproliferative, and immunomodulatory activities. The Roswell Park Memorial Institute program concerns: (*a*) large-scale production (1) and purification for HFIF, preparatory to elucidation of its primary structure and clinical investigation of its pharmacologic activity in certain tumors and in those patients with neoplasia and intercurrent viral infections, and (*b*) large-scale synthesis of mismatched ("self-destruct") inducers [$rI_n \cdot r(C_{12},U)_n$] and their clinical application in cancer (2,3). In a long-term collaboration with Prof. P.O.P. Ts'o, we have shown that these self-destruct inducers have therapeutic ratios substantially superior to other known interferon inducers. At present, approximately 35 g of $rI_n \cdot r(C_{12},U)_n$ has been prepared for clinical investigation.

Dr. Judith O'Malley of our staff is particularly concerned with molecular aspects of these inducers; specifically, she is determining which structural forms of human interferon (fibroblast, immune, or leukocyte) are synthesized following a "triggering" event with either the well-registered ($rI_n \cdot rC_n$) or the mismatched [$rI_n \cdot r(C_{12},U)_n$] duplex polymers (4).

A variety of molecular probes, developed here, have been used to separate physically the three classes of endogenous interferon molecules induced by double-stranded RNA therapy. To date, approximately 40 patients have been treated with the well-registered inducer (5), and interferon levels as well as various indices of inducer toxicity have been recorded. It may eventually be possible to assign specific biologic functions in man to each type of interferon which is induced, and such assignments are part of our long-range goal.

Our institute's current production of crude HFIF well exceeds 1×10^9 units/month (1), under the direction of S. Leong, Project Manager. Since the adult dose may be greater than 1×10^6 units/60 kg/day, this production pattern reflects an ability to carry on a set of interlinked clinical protocols simultaneously (table 1). A new technique, developed with our colleague Dr. Eugene Sulkowski, termed *tandem chromatography* (6), has been applied to HFIF and results in a purification of several thousand fold. The two major segments of our current clinical program (purified interferon and interferon inducer) represent 10 years of program effort, of which 8 years were spent in developing techniques and applying the various concepts in tissue culture and small animal systems.

These clinical studies also provide an opportunity to determine the possible immunomodulation properties of purified fibroblast interferon and its self-destruct inducers. To this end, a close collaboration has been developed with Drs. Ernest Borden and Joyce Zarling of the Immunology Research Center (University of Wisconsin Cancer Center, Madison). We have recently determined that purified HFIF

[1]Supported in part by Public Health Service grants CA-14801 and CA-15502 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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TABLE 1.—Clinical protocols

Protocol No.	Protocol description	Collaborating Roswell Park Memorial Institute departments
I	$rI_i \cdot rC_n$ in V-Z infection	Pediatrics and Medicine A and B
II	$rI_n \cdot rC_n$ in diffuse pulmonary infiltrates	Pediatrics and Medicine A and B
III	HFIF in children after V-Z exposure	Pediatrics
IV	HFIF in melanoma and breast metastases (intralesional therapy)	General Surgery, Breast, and Medicine A and B
V	HFIF in chronic active hepatitis	Multicenter
VI	HFIF in primary hepatoma or chronic hepatitis syndrome	Multicenter
VII	$rI_n \cdot r(C_{12}U)_n$ in certain of the above-mentioned diseases	

augments both NK and T killer cell activity in vitro.[3] Special excitement exists for extending these observations to the clinic. For example, we plan to evaluate T and B cell function, following interferon therapy, in patients with a chronic viral infection (eg, hepatitis B) (7) as well as in patients with various neoplasias. Such comparative studies may eventually allow us to determine more clearly the therapeutic potential of the particular biologic property of the interferon molecule.

Recent clinical study by Merigan et al with human leukocyte interferon strongly suggests its efficacy both in the treatment of herpes zoster in patients with cancer (8) and in the treatment of varicella in children with cancer (9).

[3]Zarling JM, Sosman J, Borden EC, et al. Enhancement of cytotoxic T cell responses by purified human fibroblast interferon. J Immunol 121:2002–2004, 1978, and unpublished data.

It will be particularly valuable therefore to compare HFIF and human leukocyte interferon for therapeutic efficacy in various human neoplastic and viral diseases, as well as in disorders of the immune system. Since human interferons from these two cell sources differ in certain physical (10), antigenic, and possibly biologic characteristics, certain diseases may be more responsive to a specific molecular form of human interferon.

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Human Fibroblast Interferon in Human Neoplasia: Clinical and Laboratory Study [1]

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SUMMARY

Human fibroblast interferon (HFIF) produced on a large scale from normal diploid cell strains was highly purified and then evaluated as to its safety for clinical investigation. The selective antiproliferative activity of HFIF was observed in vitro against certain human malignant cell lines and in vivo against human bladder tumors grown in nude mice. Direct injections of HFIF into metastatic melanoma lesions of two patients resulted in either the disappearance of malignant cells or the significant reduction in tumor volume.

[Cancer Treat Rep 62:1899–1906, 1978]

Human fibroblast interferon (HFIF) is a glycoprotein with antiviral, antiproliferative, and immunomodulatory activities. We describe herein the recent development of a large-scale production purification facility for the purpose of obtaining sufficient quantities of purified and safety-tested HFIF for clinical investigation. Each of these three biologic functions (inhibition of viral and cell growth and immunomodulation) is being evaluated in patients with various neoplastic diseases. Certain human tumors are shown to have a preferential sensitivity to the antiproliferative effect of HFIF. The patient with neoplastic disease may possess, in addition to this tumor, a variety of immunologic lesions as well as a predilection for viral infections. Thus, each of the three biologic functions of HFIF might favorably alter either the primary neoplastic disease or some of its serious secondary effects.

[1] Supported in part by Public Health Service grants CA-14801 and CA-15502 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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[3] We thank Drs. E. Sulkowski and A. Mikulski for comments on this manuscript and acknowledge their input into the development of human fibroblast interferon purification procedures. We are also grateful to L. A. Di Berardino, D. Donovan, L. Fletcher, and R. Heinaman for their excellent technical assistance.

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Large-Scale Production of Human Fibroblast Interferon

Mass production of HFIF from human foreskin fibroblasts for clinical investigation was initiated in our laboratories in the spring of 1976. The interferon is "superinduced" by treating the cells with $rI_n \cdot rC_n$, actinomycin D, and cycloheximide. Since May 1977, we have been producing up to 10^9 reference units of crude HFIF per month. A detailed description of our methods to produce HFIF, our characterization of isolated cell strains, and our overall quality control scheme is provided in a recent publication (1).

Several cell strains for HFIF production have been established in our laboratory and ample stocks of cells at the 6th passage level are kept frozen in liquid nitrogen (90–100 ampules with 2×10^6 cells/cell strain). A total of 12 cell strains which meet the Food and Drug Administration (FDA) requirements are available in our cell bank and are ready to be used for large-scale production of HFIF. Our calculations indicate that the size of our cell stock will provide an adequate supply for full-scale operation at the present level for many years to come.

The 12 strains of human foreskin fibroblast (derived from the foreskins of normal 3-day-old boys) for large-scale HFIF production are elaborating on the average of 26,000–30,000 reference units of interferon per ml of harvested medium. Properties of these cell strains are typical of normal diploid human fibroblasts. They have fibroblast morphol-

ogy, are fully contact-inhibited, and produce smooth and uniform monolayers; the saturation density on minimum essential medium (MEM) + 10% fetal calf serum is 6–7 × 10^4 cells/cm^2. The in vitro lifespan of these cells is between 55 and 67 population doublings. Karyotypic analysis indicates that the distribution of the chromosome number is normal diploid for human male karyotype, ie, 46, XY. These cell strains are free of detectable adventitious agents such as viruses, mycoplasma, bacteria, and fungi.

Use of the best strain of foreskin fibroblasts available assures us of maximum yields of HFIF with the highest efficiency. Further improvements for increasing HFIF yield in the superinduced cell cultures to reduce costs (eg, double harvesting, modification of production medium, superinduction procedures, etc) are being regularly introduced.

For large-scale interferon production, one fresh ampule of cell seed stock is removed (every 10 weeks) from liquid nitrogen storage tanks and propagated by 2:1 (twice a week) subcultures up to the 18th passage level, when sufficient numbers of cells become available to form monolayer cultures in 128 large roller bottles (1585 cm^2 growth area). From this time on, for the next 12 weeks, interferon production takes place (four times a week, superinduction of cells in 16 bottles). Also, four times a week cells from 16 bottles are subcultured (2:1) in order to maintain a constant number of vessels with approximately 1 × 10^8 cells which are 7 days old and suitable for interferon superinduction. The cells are phased out of production between the 30th and 33rd passage (population doubling level). At this time, a cell stock newly built up, at the 18th passage level, enters the production cycle.

Confluent monolayer cultures in roller bottles are superinduced (2,3) as follows: the culture medium in each bottle is aspirated and 100 ml of MEM containing 75 μg/ml of $rI_n \cdot rC_n$ (Miles Laboratory) and 60 μg/ml of cycloheximide (Upjohn Co) are added per bottle. The cultures are rolled for 220 minutes at 34°C prior to the addition of actinomycin D (Merck, Sharp and Dohme), to a final concentration of 0.75 μg/ml. After 110 minutes, the induction medium is removed and the cells are washed three times with 200 ml of pre-warmed Eagle's balanced salt solution. Next, the production medium (MEM plus 5% heat-inactivated human serum) is added (100 ml), and cultures are incubated for an additional 22 hours at 34°C. At this time, the cell culture medium containing interferon is harvested, pooled, centrifuged, and frozen at –90°C. The average yield of the crude HFIF is approximately 30,000 international reference units (RU)/ml of harvested medium. Interferon assays are performed as described (4), and interferon titers are expressed in RU/ml according to standard G-023-901-527, Research Resources Branch, National Institutes of Health.

Large-Scale Purification of Human Fibroblast Interferon

Purification of large amounts of HFIF involves utilization of concanavalin A (Con-A)-Sepharose (5,6) and Phenyl-Sepharose CL-4B column chromatography.[4] A crude preparation of 5–7 liters (weekly production output) of HFIF is charged simultaneously on a battery of Con-A columns previously equilibrated in 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl (6). HFIF is bound to the matrix of the column together with some contaminating glycoproteins by means of carbohydrate recognition and hydrophobic interaction. Over 90% of the total protein passes through in the breakthrough fraction. After the material is applied, the column is washed with phosphate-buffered saline (PBS) (0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4), followed by elution with 0.1 M α-methyl-*d*-mannoside (α-MM) which removes most of the glycoproteins. Displacement of the HFIF from the column is achieved with 50% ethylene glycol in 0.1 M α-MM (vol/vol). This procedure gives an average 2000-fold purification resulting in specific activity of between 1 and 3 × 10^7 units/mg of protein. In the second step of our large-scale purification procedure, the HFIF (in 40% ethylene glycol) is applied to a phenyl-Sepharose CL-4B column. The HFIF binds to phenyl-Sepharose CL-4B by hydrophobic interaction, and, following an extensive wash, is displaced by 75% ethylene glycol in PBS. Such a procedure results in a highly purified and concentrated preparation with specific activity of up to 5 × 10^7 RU/mg of protein.

Following addition of human serum albumin (HSA) (3 mg/ml) for enhancement of interferon stability and extensive dialysis against PBS, pH 7.4, the HFIF preparation is filtered (0.22 μ), freeze-dried, and stored for further use (table 1). This product undergoes extensive testing in vitro and in experimental animals to meet FDA requirements for biologic products intended for human use (see below). Alternatively, the product obtained from phenyl-Sepharose CL-4B column is stored in 25%–50% ethylene glycol at –90°C in polypropylene containers. We found that under these conditions only minimal losses of activity occurred over a 2-month period.

[4]Mikulski AJ, Horoszewicz JS, Sulkowski E, et al. Personal communication, Interferon Scientific memoranda, Oct 1976.

TABLE 1.—Lyophilized product characterization

Each vial contains—	
HFIF purified to specific activity of approximately 2×10^7 RU/mg of protein	Approximately 500,000 RU
Normal HSA, USP Squibb, added as HFIF preservative	3.46 mg
Sodium chloride	8.5 mg
Sodium phosphate	2.4 mg

An important advantage of the purification procedures developed by our own group is the absence of exposure of the interferon product to any denaturing (partial or complete) condition which might affect the native conformational state of this glycoprotein. Thus, any exposure to chaotropic salts (as thiocyanates) or detergents (as sodium dodecyl sulfate) has been carefully avoided throughout the purification of HFIF. Exposure, even transient, to such denaturing agents carries the theoretic concern for irreversible alteration of the protein molecule. Such alteration could result in unexpected biologic effects, such as modification of antigenicity, etc, on repeated administration, as well as undesirable biochemical changes, such as molecular aggregation, etc.

Quality Control and Safety of Human Fibroblast Interferon After Purification

During the second half of 1977, our laboratories purified $> 850 \times 10^6$ RU of HFIF to a specific activity of 2×10^7 RU/mg of protein using the above-described procedures. After lyophilization, this product was evaluated following the Code of Federal Regulations 21 (FDA) 1976, parts 600–1299, on general safety (table 2), pyrogenicity (table 3), sterility, and toxicity for young rats and mice (table 4 and fig 1). Our HFIF passed all these tests easily.

Ten female Sprague-Dawley rats and ten female Swiss mice (4 weeks old) received nine ip injections (biweekly) of purified HFIF containing HSA over a period of 28 days. The weekly dosages per animal were as follows:

Rats, 260,000 RU of HFIF with 1.76 mg of HSA
Mice, 48,000 RU of HFIF with 0.32 mg of HSA

The dosages on the basis of body weight correspond to a daily dose of between 17 and 24 million units of HFIF for a 60-kg man. The same number of matched control animals received injections of sterile saline using a similar schedule. The weight of each animal was measured weekly. Four days following the last injection, all animals were killed. At Day 0, 7, 14, 21, 28, and 32, the weight of each animal was recorded. Four days following the last HFIF injection, blood and serum from all animals were collected for appropriate testing. The animals were killed and autopsied, the organs (lungs, liver, spleen, kidneys, and thymus) were fixed in 10% formalin, and H & E-stained sections were prepared.

Pathology Report

Sections of liver, kidney, lung, spleen, and thymus of all rats and mice receiving either HFIF injections or saline were examined. No pathologic changes related to HFIF injections were seen.

FIGURE 1.—Toxicity tests in young rats and mice.

TABLE 2.—General safety test*

Animal	Weight of animals (g) Before injection	After 7 days	Gain
Guinea pig			
1	365	420	55
2	392	450	58
Mouse			
1	19.5	22.0	2.5
2	21.5	24.0	2.5
3	20.0	22.5	2.5
4	21.0	23.5	2.5

*12 vials of the final product were reconstituted in 12 ml of water for injection. This was injected ip into 2 guinea pigs (5 ml each) and 4 mice (0.5 ml each). The animals were observed for 7 days and weighed. All animals survived the test period, did not exhibit any response which was nonspecific or unexpected, and gained weight. These dosages on the basis of body weight correspond to a dose of 400–800 million units for a 60-kg man.

In addition, we have determined that intradermal injections of 90,000 RU in 0.2 ml in nine human volunteers produced only mild reactions limited to faint erythema without detectable induration (table 5). Specifically, no delayed-type hypersensitivity reactions similar to those reported by De Somer et al (7) were observed.

These tests are the final steps in our scheme of quality control of HFIF (1). Thus, we have in practice completed the entire quality control scheme which started with the evaluation of suitable cell strains, continued through the production and purification of interferon, and extended to the safety testing of HFIF in animals and human skin.

Current interpretation of FDA guidelines indicates that human interferon obtained from leukocyte or lymphoblastoid cultures will probably not be allowed widespread clinical application because of possible contamination with slow viruses or with small fragments of the EB virus genome (nucleic acid hybridization being unable to detect small potentially transforming sequences, etc). Therefore, it is generally agreed that the natural product, interferon, derived from normal diploid fibroblasts, may be unique from the family of human interferons in meeting all safety requirements.

Antiproliferative Effects of Highly Purified Human Fibroblast Interferon

The antiproliferative activity in vitro of highly purified HFIF against several established human

TABLE 3.—Pyrogenicity test*

Rabbit No.	Weight (g)	Injection (ml)	Injection temperature (°C)	Postinjection temperature (°C)			Greatest rise
				1 hr	*2 hrs*	*3 hrs*	
G25	2827	10	39.4	39.0	39.2	39.1	
G26	2725	10	39.5	39.2	40.0	39.4	
G27	3189	10	39.5	39.0	39.4	39.4	
				4 hrs	*5 hrs*	*6 hrs*	
				39.2	39.1	39.3	0.0
				39.6	39.9	39.7	0.5
				39.2	39.4	39.4	0.0

*3 rabbits were injected with 417,000 RU of HFIF and their body temperature was monitored for 6 hrs. These dosages on the basis of body weight correspond to a dose of between 7.8 and 9.2 million units of HFIF for a 60-kg man. HFIF did pass the test for absence of pyrogens.

tumor cell lines was tested as described (8). These cell lines were obtained from Dr. J. Fogh of the Memorial Sloan-Kettering Cancer Institute. The proliferation of 5959 (osteogenic sarcoma), RT-4 (transitional cell carcinoma), and DAUDI (Burkitt's lymphoma) cell lines was inhibited > 50% by 100 RU/ml of purified HFIF. In contrast, a concentration of > 1000 RU/ml of HFIF was required for 50% growth inhibition of cell lines A204 (rhabdomyosarcoma), HT-29 (adenocarcinoma of the colon), and MeWo (malignant melanoma). This lower susceptibility to the antiproliferative effect of HFIF was also observed when normal human diploid fibroblasts were tested. Thus, it is apparent that HFIF has *selective* antiproliferative activity in vitro against certain (osteogenic sarcoma, transitional cell carcinoma, and Burkitt's lymphoma) established human tumor cell lines.

In addition, we have found that HFIF can modulate [^{3}H]TdR incorporation in cultures of epithelial and fibroblastic cells derived from benign prostatic hyperplasia. HFIF (100–1600 RU/ml) added for 48 hours to actively proliferating prostatic epithelial explants or subconfluent monolayers of prostatic fibroblasts resulted in a strong dose-dependent reduction in the number of [^{3}H]TdR-labeled nuclei (by autoradiography) as well as a decline in the total radioactive counts incorporated. Prostatic epithelial cells were found to be between twofold and tenfold more susceptible to inhibition of [^{3}H]TdR incorporation by HFIF than prostatic fibroblasts (fig 2). These observations suggest a selective antiproliferative effect, hitherto undetected, of HFIF against epithelial versus fibroblastic cells.

Our studies on the antiproliferative effects of HFIF were extended to the use of athymic nude mice as hosts for human malignant cells. Nude mice (six to ten animals per group) were injected sc with DAUDI, MeWo, A-204, or RT-4 cells (5×10^6 cells/animal). These mice were treated daily with 20,000 RU of HFIF or a corresponding amount of diluent over a period of 6 weeks. A comparison of the mean tumor volumes in mice treated with HFIF or diluent served as indices of HFIF effectiveness in vivo. The growth rate of tumors induced by injection of RT-4 cells was significantly reduced (over fourfold) in HFIF-treated mice (table 6). Tumors induced by injection of MeWo, A-204, and DAUDI cells did not respond to HFIF treatment. HFIF was not found to be effective against DAUDI tumors in nude mice despite the demonstrated susceptibility of these cells to the antiproliferative activity of HFIF in vitro. However, the in vivo susceptibility of RT-4, MeWo, and A-204 tumors to HFIF correlated well with the in vitro antiproliferative activity of HFIF for these cells.

Finally, in the clinic, the highly purified HFIF (5×10^5 RU/day) was injected directly into the cutaneous or subcutaneous metastatic lesions of two patients with malignant melanoma. A vehicle control with the same pH, osmolality, and human albumin content but without HFIF was injected into other lesions of similar size. In the first patient, the cutaneous lesion (5 mm in diameter) injected with HFIF for 14 consecutive days disappeared grossly and no tumor cells were evident upon microscopic examination of serial histologic sections of the entire excised area. Uninjected lesions and lesions injected with vehicle control contained typical masses of melanoma cells. The second patient with multiple subcutaneous melanoma metastatic nodules was treated for 30 days. All untreated nodules, as well as a control nodule injected with the vehicle, continued to grow and doubled their volumes within a month. In contrast, the lesion treated daily with HFIF was reduced to one fourth of the original volume (fig 3). However, upon microscopic examination, tumor cells were still present.

TABLE 4.—Results of safety testing of HFIF in young rats and mice

Animal	Mean values: HFIF injected ± SE	Mean values: Controls ± SE	% of controls	Difference between means	Statistical significance of difference
Rat					
Body weight (g)					
Day 0	105.6 ± 1.0	102.1 ± 0.9	103	3.5	NS*
Day 7	141.9 ± 1.2	135.1 ± 2.1	105	6.8	NS
Day 14	165.2 ± 2.8	161.1 ± 2.7	103	4.1	NS
Day 21	183.5 ± 3.4	179.4 ± 2.9	102	4.1	NS
Day 28	203.2 ± 4.3	193.5 ± 3.3	105	9.7	NS
Day 32	208.4 ± 4.6	196.4 ± 3.5	106	12.0	NS ($t = 2.07$)
Weight gain (Days 0–32)	102.6 ± 4.4	94.3 ± 3.3	109	8.3	NS ($t = 1.51$)
Hemoglobin (g/100 ml)	15.01 ± 0.16	13.81 ± 0.53	109	1.2	NS ($t = 2.18$)
Wbc count × 10^3	10.6 ± 0.56	10.51 ± 1.24	101	0.09	NS
Rbc count × 10^6	6.84 ± 0.11	6.65 ± 0.19	103	0.19	NS
Platelet count × 10^5	9.99 ± 0.29	10.87 ± 0.84	92	0.88	NS
Activated partial thromboplastin time in seconds	20.8 ± 0.46	21.2 ± 0.61	98	0.4	NS
Prothrombin time in seconds	14.3 ± 0.22	14.3 ± 0.27	100	0.0	NS
Serum biochemistry					
Glucose (mg/100 ml)	123.3 ± 2.64	138.2 ± 14.37	89	14.9	NS
Urea nitrogen (mg/100 ml)	23.65 ± 1.04	23.09 ± 1.27	102	0.56	NS
Creatinine (mg/100 ml)	0.66 ± 0.03	0.36 ± 0.05	183	0.3	$P < 0.01$†
Uric acid (mg/100 ml)	2.29 ± 0.15	2.83 ± 0.18	81	0.54	$0.02 < P < 0.05$
Cholesterol (mg/100 ml)	86.3 ± 13.5	81.6 ± 4.3	106	4.7	NS
Alkaline phosphatase (units/ml)	64.1 ± 3.3	63.0 ± 6.7	102	1.1	NS
SGOT (units/ml)	60.7 ± 3.6	139.0 ± 32.6	44	42.2	NS
SGPT (units/ml)	13.0 ± 1.0	22.9 ± 6.1	57	9.9	NS
LDH (units/ml)	207.8 ± 31.6	805.1 ± 192.5	26	597.3	$P < 0.01$†
Total bilirubin (mg/100 ml)	0.16 ± 0.016	0.15 ± 0.016	107	0.01	NS
Albumin (g/100 ml)	4.46 ± 0.04	4.26 ± 0.23	105	0.2	NS
Total protein (g/100 ml)	6.32 ± 0.09	6.69 ± 0.32	95	0.37	NS
Mice					
Body weight (g)					
Day 0	18.45 ± 0.26	18.55 ± 0.23	99.5	0.1	NS
Day 7	19.2 ± 0.8	19.5 ± 0.4	99	0.3	NS
Day 14	20.5 ± 0.56	21.6 ± 0.22	95	1.1	NS
Day 21	22.4 ± 0.82	22.5 ± 0.35	99.5	0.1	NS
Day 28	23.7 ± 0.87	24.2 ± 0.21	98	0.5	NS
Weight gain (Days 0–28)	5.2 ± 0.86	5.6 ± 0.15	93	0.4	NS
Wbc count × 10^3	11.93 ± 0.73	10.67 ± 0.87	112	1.27	NS
Rbc count × 10^6	9.02 ± 0.27	8.85 ± 0.19	102	0.17	NS
Platelet count × 10^5	11.4 ± 0.8	10.6 ± 1.0	107	0.7	NS
Serum biochemistry					
Glucose (mg/100 ml)	82.2 ± 11.8	46.8 ± 8.2	175	35.4	NS
Urea nitrogen (mg/100 ml)	29.7 ± 1.9	29.7 ± 0.7	100	0.0	NS
Creatinine (mg/100 ml)	0.37 ± 0.13	0.42 ± 0.07	88	0.05	NS
SGOT (units/ml)	100.0 ± 10.3	120.8 ± 31.4	83	20.8	NS
SGPT (units/ml)	21 ± 1.6	29 ± 3.5	72	8.0	NS

*NS = not significant.

†Due to partial hemolysis in 4 sera.

TABLE 5.—Human skin reactivity to purified HFIF (90,000 RU/0.2 ml)

Subject No.		Immediate reaction							
		< 6 hrs		24 hrs		48 hrs		72 hrs	
		HFIF	Control	HFIF	Control	HFIF	Control	HFIF	Control
1	I*,†	12 × 12	—	—	—	—	—	—	—
	E‡	40 × 40	—	18 × 35§	—	20 × 45§	—	±¶	—
2	I	—	—	—	—	—	—	—	—
	E	30 × 30	—	—	—	—	—	—	—
3	I	—	—	—	—	—	—	—	—
	E	18 × 18	—	18 × 20§	—	25 × 25§	—	25 × 25§	—
4	I	5 × 5	—	—	—	—	—	—	—
	E	30 × 30	30 × 30	20 × 20§	—	—	—	—	—
5	I	—	—	—	—	—	—	—	—
	E	20 × 20	20 × 20	20 × 20	20 × 20	±¶	±¶	—	—
6	I	—	—	—	—	—	—	—	—
	E	—	—	—	—	—	—	—	—
7	I	—	—	—	—	—	—	—	—
	E	—	—	—	—	—	—	—	—
8**	I	—	—	—	—	—	—	—	—
	E	5 × 5	—	—	—	—	—	—	—
9††	I	—	—	—	—	—	—	—	—
	E	7 × 7	—	—	—	—	—	—	—

*Measured in mm.
†I = induration.
‡E = erythema.
§Very faint.
¶Discolored.
**Before HFIF treatment.
††After HFIF treatment for 30 days.

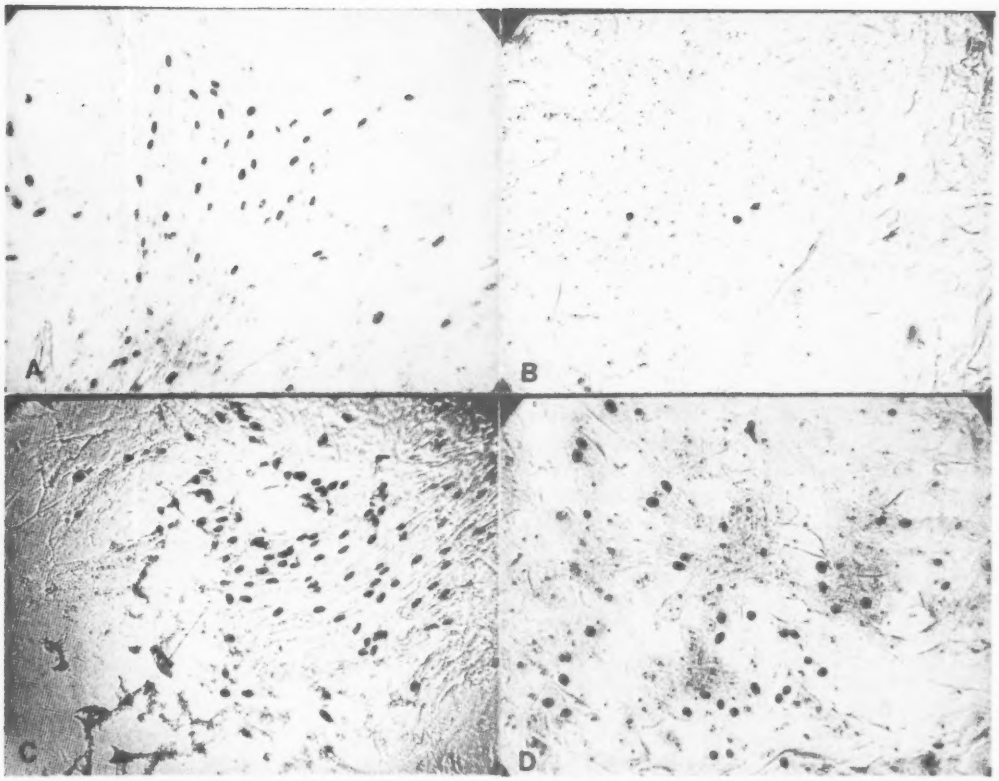

FIGURE 2.—Autoradiographs showing [^{3}H]TdR incorporation in human prostate cells in the presence and absence of treatment with interferon. Actively growing cells were treated with interferon-containing or control medium. Interferon was purified as described under large-scale purification. After a 48-hr incubation, cells were pulse-labeled with [^{3}H]TdR and prepared for autoradiography. Epithelial cells in the (A) absence and (B) presence of interferon (1600 RU/ml). Fibroblasts in the (C) absence and (D) presence of interferon (1600 RU/ml).

TABLE 6.—Human tumors in nude mice treated with HFIF

Injected cell line	Tumor	Tumor volume at 30 days as % of controls ("mock"-treated mice) ± SE
RT-4	Bladder cancer	24.9 ± 7.5
MeWo	Melanoma	78.6 ± 31.2
DAUDI	Burkitt's lymphoma	87.0 ± 14.2
A-204	Rhabdomyosarcoma	151.5 ± 39.2

In both patients, the metastases injected with HFIF were heavily infiltrated with lymphocytes. No such infiltrations were observed in control lesions. No fever or any other side effects associated with HFIF injections were observed.

In summary, our current results obtained with HFIF tested against human malignant cells in vitro, in the nude mice, and in patients strongly suggest that this glycoprotein may shortly find a practical application in therapy for selected malignancies.

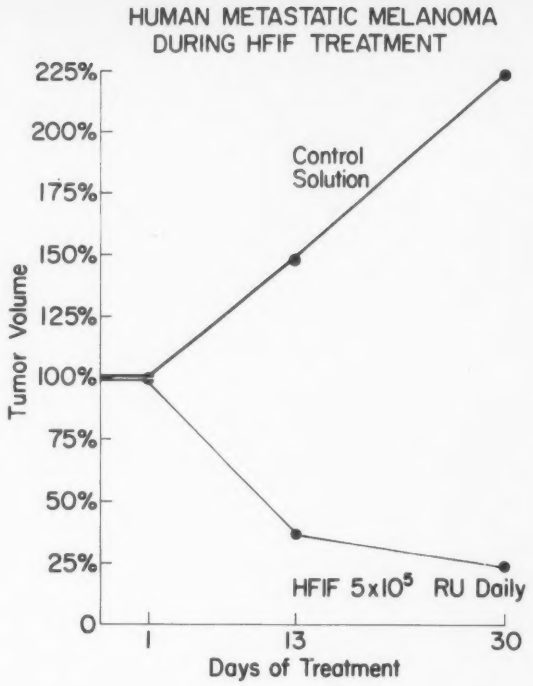

FIGURE 3.—Intralesional injection of HFIF in subcutaneous melanoma. Lesions were treated daily with direct injection of HFIF (5×10^5 RU) or the vehicle alone (contents described in table 1) for 30 consecutive days.

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Phase I–II Trials of Poly IC Stabilized With Poly-L-Lysine

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SUMMARY

Poly IC, stabilized with poly-L-lysine and carboxymethyl cellulose (poly ICLC), resists hydrolysis by primate serum (unlike the parent compound), induces high levels of serum interferon, and is effective in acute viral infections of subhuman primates. In a phase I–II clinical trial, poly ICLC was given iv in 15 daily doses of 0.5–27.0 mg/m^2 to 19 patients with various solid tumors and to six patients with acute leukemia (1–65 years of age). At least three complete trials were conducted at each of six dose levels. Toxic reactions included fever (in 100% of trials), nausea (44%), hypotension (28%), thrombocytopenia and leukopenia (68%), erythema (12%), and polyarthralgia plus myalgia (16%). Hypotension and arthralgia-myalgia were related to dose level and/or magnitude of interferon induction, but other toxic manifestations were not. Poly ICLC induced significant serum interferon levels in 76% of trials, and the correlation between dose and peak interferon titer was linear. The maximum tolerated dose for all patients at a given drug dose was 12 mg/m^2; at this dose, the mean peak interferon titer was 1940 reference units/ml. At a dose of 18 mg/m^2, the mean peak interferon titer was 4473 reference units/ml, but severe myalgia and arthralgia were intolerable in at least half of the patients, and most had significant hypotension. At a dose of 27 mg/m^2, one patient had acute renal failure. At high doses, iv poly ICLC also induces interferon in the cerebrospinal fluid.

[Cancer Treat Rep 62:1907–1913, 1978]

The synthetic double-stranded RNA, poly IC, is an effective inducer of interferon in vitro and in vivo in mammalian cells, including those derived from man (1–6). Exposure of such cells to microgram amounts of this agent results in significant resistance to the replication of RNA and DNA viruses (4,6). Moreover, poly IC has proven useful in prophylaxis and treatment of lytic viral infections in certain animals (3,7,8).

As with interferon (9,10), there is evidence that poly IC may have, in addition to an antiviral role, an antineoplastic mechanism of action. The compound has been used successfully to inhibit the growth of various transplantable rodent tumors, including those known to be induced by virus and those which are apparently spontaneous in origin (11–14). Poly IC is also effective in preventing the occurrence of malignancy after inoculation of rodents with purified oncogenic viruses (13,15). The polynucleotide has at least three activities which might relate to its antitumor activity: (*a*) induction of interferon, (*b*) nonspecific stimulation of immune mechanisms, and (*c*) nonspecific and specific effects on cell membranes and on macromolecular synthesis (1,6,10–12,16).

We previously examined the parent compound, poly IC, in a phase I–II clinical trial (17). While the complex had been a good interferon inducer as well as an effective antiviral and antitumor agent in rodents, it proved to be a poor interferon inducer in man, had no detectable antitumor or antiviral effect, and was not clinically useful at any dosage. It is known that primate serum contains a high level of hydrolytic activity (nuclease) against poly IC; thus, rapid hydrolysis appears to be responsible for the absence of clinical activity (18).

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Recently, a stabilized poly IC complex has been prepared which appears to resist hydrolysis by serum nuclease and is an effective interferon inducer in subhuman primates (19). The stabilized product is a complex of high molecular weight poly IC and low molecular weight poly-L-lysine (poly ICLC). The preparation, which also contains carboxymethyl cellulose, is eight to ten times more resistant to hydrolysis by pancreatic ribonuclease than is the parent poly IC. When injected iv into monkeys or chimpanzees, significant serum interferon levels have been obtained (19,20). Rhesus monkeys have been protected against certain viral infections using poly ICLC (19,20). Extensive toxicologic studies have been completed employing rodents, cats, rhesus monkeys, and chimpanzees. Rhesus monkeys were given a dose of 36 mg/m² of poly ICLC daily for 6 days and then three times weekly for the following 14 weeks with no apparent toxicity or gross pathology (Stephen E, personal communication). Chimpanzees received 60–90 mg/m² in one iv dose with a transient elevation in SGOT and slight thrombocytopenia and leukopenia, but no other chemical or hematologic abnormalities. Four of four chimpanzees given this dose every other day for 6 weeks developed chemical hepatitis which was thought to be due to the carboxymethyl cellulose component (20). The LD50 in the rat was 140 mg/m² with death due to congestive heart failure (Rakieten N, personal communication). In all animal studies to date, the poly ICLC preparation has been pyrogenic.

We report here the results of initial clinical trials of poly ICLC in cancer patients.

METHODS

Patient Selection and Drug Schedule

Poly ICLC was administered to 25 patients (1–65 years of age; 21 males and four females) who had solid tumors or acute leukemia refractory to established therapy and who did not require any additional treatment for the ensuing 21 days (table 1). At least three complete trials were conducted at each of six dose levels within the range of 0.5–27.0 mg/m². The drug was given iv in 50 ml of normal saline over a 60-minute period on the first day of the study, followed by a 7-day rest period to permit evaluation of any unanticipated toxicity. Following the rest period, the drug was given every day for the next 2 weeks. Thus, the total study period was 21 days. Patients were given no other chemotherapy for at least 10 days prior to this trial and for at least 3 weeks following conclusion of the trial.

TABLE 1.—Patients entered in initial clinical trial of poly ICLC

Disease	No. of patients
Acute leukemias (all variants)	6
Sarcomas (soft tissue or bone)	6
Lymphomas (non-Hodgkin's)	3
Carcinomas	
Lung	4
Kidney	2
Liver	1
Prostate	1
Melanoma	1
Carcinoid	1
Total	25

Determination of Toxicity

Toxicity was evaluated by close observation of clinical parameters and by frequent evaluation of the hemogram as well as renal and liver functions. Coagulation studies were performed before the first dose of poly ICLC and were repeated 24 and 48 hours after the initial dose. Thereafter, these studies were done weekly for the duration of each patient's study. Cbcs and differential counts were performed immediately before the drug was given and 4 hours after the first dose; thereafter, they were obtained 3 times weekly. Serum chemistry values were determined immediately prior to the first dose and twice weekly thereafter.

Tumor Response

Appropriate serial studies (roentgenograms, radioisotope scans, bone marrow examinations, tumor markers, etc) were carried out to assess tumor response, with weekly estimates of tumor size. In patients with solid tumors, response was defined as a $\geq 50\%$ reduction in the greatest diameter of an evaluable tumor or measurable radiologic density. In patients with acute leukemia, response was defined as a significant reduction in the percentage of blast cells in the bone marrow and/or peripheral blood.

Drug Formulation

Poly-L-lysine was obtained from Sigma Chemical Co, St. Louis, Mo. Poly IC was obtained from PL Laboratories, Milwaukee, Wis. Sodium carboxymethyl cellulose is a product of the Hercules Powder Co, Wilmington, Del. A solution of soluble complex (in pyrogen-free 0.85% sodium chloride)

was prepared as previously described (19). The final preparation contained 2 mg/ml of poly IC and 1.5 mg/ml of poly-L-lysine in 0.5% carboxymethyl cellulose.

Interferon Assay

Serum interferon concentrations were determined on 5-ml samples collected immediately prior to starting the drug infusion on Day 1; subsequent samples were collected 8 and 24 hours after the start of the 1-hour infusion. These studies were repeated in the same sequence each day from Day 8 to 21. Cerebrospinal fluid (CSF) was also collected (3–5 ml) for interferon assay immediately prior to the first infusion of poly ICLC and 4 hours after the start of the infusion. CSF was also collected on Day 8 in the same sequence. To determine interferon titers, we employed the VSV-CPE protection test (21). One unit of interferon is defined as that amount which, when suitably incubated with proper test cells, will cause a reduction of 0.5 $\log_{10}$ in the yield of a suitable sensitive challenge virus (VSV) as compared with the yield from cells not treated with interferon.

RESULTS

Interferon Induction

Table 2 lists the mean peak serum interferon titers attained following administration of poly ICLC at a dose range of 0.5–27.0 mg/m². In preliminary trials, with serum sampled every 4 hours for 24 hours following each dose, peak levels were noted in the sample collected 8 hours following the first dose of poly ICLC; the tabulated data therefore represent the mean serum interferon titers at that time for all patients. All patients had pretreatment levels of < 5 reference units/ml. As noted with the parent compound, poly IC, peak interferon levels dropped rapidly with continued daily exposure to the drug (2,17,22); every patient manifested a significantly lower serum level of interferon following the fourth or fifth dose. However, hyporesponsiveness was less marked with higher doses (> 7.5 mg/m²) of poly ICLC. Representative kinetic curves of interferon induction in response to the parent compound, poly IC, have been published (17), and the kinetics of serum interferon in response to all doses of poly ICLC were similar (data not shown). The mean peak serum interferon titers attained in this trial with doses of ≥ 7.5 mg/m² were extraordinarily high, exceeding those usually noted with naturally occurring viral infections. Poly ICLC induced significant serum levels in 76% of the trials, and the correlation between drug dose and peak interferon titer was approximately linear. The maximum tolerated dose for all patients at a given drug dose was 12 mg/m². At this dose, the mean peak interferon titer was 1940 reference units/ml. At 18 mg/m², the peak serum interferon in one patient was 15,000 reference units/ml (mean of all patients, 4473), but the side effects of the drug were intolerable in at least half of the patients so treated, and most had significant hypotension. The peak serum interferon titers were not significantly higher at 27 mg/m² than at 18 mg/m², and one patient at the highest dose level had acute renal failure not explicable by any mechanism other than exposure to this drug.

TABLE 2.—Mean peak serum interferon titers after treatment with poly ICLC

Dose level (mg/m²)	Mean reference units/ml* (range)
0.5	15 (0–25)
2.5	15 (0–25)
7.5	198 (25–250)
12.0	1940 (200–5000)
18.0	4473 (600–15,000)
27.0	5820 (2000–10,143)

*VSV-CPE assay (international reference units); 8-hr sample after first dose; ≥ 3 trials at each level.

Poly ICLC also induced interferon in the CSF when given iv. However, the peak interferon titers attained were considerably less than those noted in the serum. At the maximum tolerated dose in this study, 12 mg/m², the mean peak CSF interferon titer was 34 reference units/ml. In contrast to titers detected in the serum, there was a tenfold increment in the CSF interferon titer between the dose levels of 18 and 27 mg/m². Of the samples collected, the 8-hour sample following the first dose of poly ICLC demonstrated the highest titer in all patients.

Of the dose levels employed in this study, 7.5 mg/m² appears to be the threshold below which significant levels of serum interferon were not induced. Even at higher dose levels however, a very broad range of interferon titers was found, suggesting individual variation in response to this drug or its metabolism. To an extent, this variation reflected the peripheral wbc count, although the correlation was not consistent.

Toxicity

The most frequently encountered toxic reaction in this study was fever, which occurred in all of the 25 clinical trials (table 3). The mean peak temperature

TABLE 3.—Poly ICLC toxicity*

Manifestation	No. of patients with effect/ total No. treated (%)
Fever	25/25 (100)
Nausea	11/25 (44)
Thrombocytopenia and leukopenia	17/25 (68)
Hypotension†	7/25 (28)
Syndrome of erythema, polyarthralgia, and polymyalgia†	4/25 (16)
Renal failure†	1/25 (4)
Trial aborted‡	5/25 (20)

*Maximum tolerated dose, 12 mg/m².

†Related to dose level and/or magnitude of interferon induction.

‡Hypotension, 3 cases; renal failure (27 mg/m²), 1 case; serum sickness(?), 1 case.

elevation was 1.5°C, which usually occurred 4–8 hours after drug administration; fever persisted for approximately 6–12 hours. No relation existed between the development of fever and the dose level or magnitude of interferon induction. Nausea occurred in 11 of 25 of the trials (44%) and was not related to dose level or magnitude of interferon induction. Modest elevations (approximately 50%) of liver enzyme levels (SGOT and SGPT) were detected in a few patients, but in each case, these could have been related to the disease per se (eg, liver metastases) or to other hepatotoxins. We observed no other liver abnormalities related to treatment. No relationship was found between the dose level or magnitude of interferon induction and the elevation of the liver enzyme levels. Thrombocytopenia and leukopenia (primarily lymphocytopenia) were demonstrable in 17 of 25 patients (68%) receiving poly ICLC, but as with abnormal liver function tests, these findings could not be attributed in many cases to exposure to the drug per se as opposed to the consequences of the underlying disease. Profound cytopenia was observed in only one patient; this patient received the 27-mg/m² dose of poly ICLC, and 1 day after the patient received the first dose, his platelet count (which had been 200,000/mm³) fell to 30,000/mm³. It seems likely that the drug does produce peripheral cytopenia due to destruction or sequestration (marrow hypoplasia was not observed). In three patients treated with poly ICLC for amyotrophic lateral sclerosis or relapsing polyneuropathy, lymphocytopenia and a biphasic granulocyte response were noted (23).

Although a small number of patients in this study (dose, 18–27 mg/m²) had a slight prolongation of the thrombin time and/or a small increase in fibrin-split products, these findings could be explained by the underlying disease as well as by exposure to poly ICLC.

The most significant toxic effects noted in this trial were hypotension, which occurred in seven of 25 patients (28%), and a syndrome of polyarthralgia and polymyalgia, with or without erythema, which occurred in four of 25 patients (16%). Hypotension and the arthralgia-myalgia-erythema syndrome did relate to dose level and/or the magnitude of interferon induction, unlike the other toxic manifestations. Hypotension (systolic and diastolic) occurred approximately 2 hours after the start of a poly ICLC infusion. It was transient and was not associated with symptoms in four patients, but was clinically significant in magnitude and duration in three others, necessitating termination of their clinical trials. Another patient, previously stable, with no history of renal or cardiovascular disease and not exposed to any other possible nephrotoxins, developed acute renal failure 72 hours after exposure to his first dose of poly ICLC at a dose level of 27 mg/m². Hypotension was not noted. The trial was terminated and the patient recovered from his episode of renal failure with no further complications. Interestingly, in those patients who experienced a decrease in blood pressure, the pressure tended to stabilize or increase during continued exposure to the drug.

The syndrome of polyarthralgia and polymyalgia (usually accompanied by erythema over the tender joints) most commonly occurred after three to five doses. This picture was consistent with a serum sickness-like reaction. Although any or all joints could be involved, those most severely involved were the proximal interphalangial joints. In three of the four patients who experienced this syndrome, symptoms and signs began to decrease while the drug was continued. However, the fourth patient, whose arthralgia primarily involved the hips and knees, developed intolerable pain. The trial was terminated and the syndrome gradually cleared. At the 18-mg/m² dose level, arthralgia was severe in at least half the patients and most had significant hypotension. In the four patients who developed the serum sickness-like syndrome, we were not able to demonstrate Farr-type polynucleotide antibodies (24). Moreover, serum complement levels were within the normal range.

In summary, the maximum tolerated dose for all patients at a given drug dose was 12 mg/m². At this dose, the mean peak serum interferon titer was 1940 reference units/ml. Five of 25 clinical trials (20%) had to be aborted because of toxicity, three due to clinically significant hypotension, one due to

acute renal failure, and one due to severe polyarthralgia-myalgia.

Tumor Response

Over a 6-week period of observation (3 weeks on the study plus a subsequent 3 weeks of observation), 23 of 25 patients demonstrated progressive tumor. However, one adult with acute undifferentiated leukemia demonstrated a brief period of stabilization, with a decrease in the number of bone marrow blast cells (from 100% to 40%), and one child with acute lymphoblastic leukemia appeared to enter a complete remission after treatment with poly ICLC. An interpretation of this child's response to the drug is complicated, however, by his exposure to chemotherapy immediately prior to the poly ICLC trial. The patient, who had had two previous relapses, was being maintained in remission on a chemotherapy regimen which included a brief exposure to cyclophosphamide. On September 28, 1977, marrow examination revealed 25% blast cells, but maintenance cyclophosphamide was given as scheduled at a dose of 5 mg/kg for 5 days. On October 14, 1977, a marrow examination revealed 50% blast cells in his bone marrow. Poly ICLC was then given at a dose of 12 mg/m^2. At the conclusion of the poly ICLC trial (November 3, 1977), this child had entered a complete remission with fewer than 5% blast cells in the bone marrow aspirate. By November 21, he had relapsed again. Poly ICLC was resumed for 15 consecutive days at a dose of 12 mg/m^2 with no leukemic response. At the conclusion of the second exposure to poly ICLC, cyclophosphamide was given at a dose of 10 mg/kg for 21 days because of the possibility that the remission apparently induced by poly ICLC was in fact associated with the maintenance dose of cyclophosphamide he had received at the end of September. However, the final trial of cyclophosphamide also failed to produce a response.

DISCUSSION

Poly ICLC, a polynucleotide interferon inducer stabilized so as to make the complex resistant to hydrolysis by serum ribonuclease, was administered at dose levels of 0.5–27.0 mg/m^2 to 25 patients with malignant diseases. At least three complete trials were conducted at each of six dose levels, and the maximum tolerated dose was found to be 12 mg/m^2. Fever was the most common side effect. No patient in this study developed significant abnormalities of liver function that could clearly be attributed to this drug, and no patient in this trial had a marked disturbance of the clotting mechanism. Peripheral cytopenia was common, but only rarely could be associated with the drug as opposed to the underlying disease.

The important toxicities revealed in this study included hypotension, acute renal failure (in one patient given the maximum dose of 27 mg/m^2), and a syndrome of polyarthralgia-myalgia primarily involving the small joints. Unlike the other side effects noted in this trial, hypotension and arthralgia-myalgia appeared to relate to the dose level and/or magnitude of interferon induction. Hypotension and joint and muscle pathology have been noted in patients given exogenous human interferon (25,26); similar complications have been detected during the administration of other polyanions, such as pyran, with or without interferon induction (27). Thus, it is not possible to say whether these toxic manifestations are due to the poly ICLC complex per se, to the high levels of endogenous interferon induced by exposure to the drug, or to both. It is also of interest that the transient granulocyte and platelet depression observed after the start of poly ICLC treatment, and improving with continued exposure to the drug, has also been noted during administration of exogenous purified human interferon (28) as well as pyran (27).

Our finding that poly ICLC consistently induces extremely high titers of interferon in the serum of man represents the first report of induction of this magnitude by an inducer which is clinically tolerable. It is probable that interferon induction would be even more efficient in patients without cancer (and neutropenia), since a preliminary trial in three patients with amyotrophic lateral sclerosis yielded serum interferon levels of 1000 reference units/ml at a dose of only 100 μg/kg (23). These results suggest that the compound should be assessed in such important human viral infections as the hepatitis B carrier state and *Herpes zoster* (25). Moreover, the fact that interferon is also induced in the CSF at higher dose levels suggests the possible utility of poly ICLC in the management of viral infections of the central nervous system such as rabies. Poly ICLC has already been employed in chimpanzees with chronic hepatitis B (20); most markers of infection disappeared but reappeared after cessation of treatment, a result similar to that obtained with purified interferon (26). This drug has also been used successfully as an adjunct to rabies vaccine in monkeys (19).

Although our report of one child with acute lymphocytic leukemia entering complete remission following exposure to poly ICLC must be inter-

preted with caution because of his prior exposure to cyclophosphamide, the finding is of particular interest in view of recent reports (29)[3] of bone marrow clearing of leukemic blast cells following administration of human leukocyte interferon. Further trials of poly ICLC as well as purified human interferon should be undertaken in childhood acute lymphocytic leukemia. Since the antitumor effect of poly IC in mice is inhibited by treatment with antimouse interferon globulin (16), it is likely that any human antileukemic effect of poly ICLC is mediated through interferon.

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Overview of the Activity of a *Brucella abortus* Preparation, Bru-Pel [1]

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SUMMARY

The properties of a nonviable, aqueous ether-extracted *Brucella abortus* preparation, Bru-Pel, are described. In addition to inducing a "virus-type" interferon response and protecting mice against challenge with otherwise lethal doses of Semliki Forest virus, Bru-Pel is demonstrated to have potent antitumor properties in mice. These antitumor effects appear to be mediated by an increase in nonspecific resistance similar to that seen with other experimental antitumor agents.

[Cancer Treat Rep 62:1915–1918, 1978]

Extraction of *Brucella abortus* with aqueous ether yields a nonviable insoluble residue, Bru-Pel, with low toxicity. This material is an effective interferon stimulus and significantly protects mice against Semliki Forest virus (SFV) infection (1). In addition to these antiviral effects, there are reports in the literature that *B. abortus* protects mice against a number of different tumors. Living cells of *B. abortus* given ip caused a 30% regression of tumors in mice given Sarcoma 180 (S180) cells sc (2) and prolonged the life of mice infected with Rauscher leukemia virus (3). Heat-killed and formalinized preparations of *B. abortus* injected ip in mice afforded significant protection against Ehrlich ascites tumors (4) as well as against L1210 leukemia cells (5).

Experiments that were carried out with Bru-Pel showed that this material given ip either before or after challenge with S180 tumor cells significantly inhibited both the development of ascites tumors and the death of the animals (6). This report will summarize some of the findings concerning the antiviral and antitumor properties of Bru-Pel.

[1]Supported by Public Health Service grant AI-32539 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health, Education, and Welfare.

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MATERIALS AND METHODS

Preparation of Bru-Pel

Cultivation of bacteria.—B. abortus 456 (ATTC-7705), a nonpathogenic strain used for antigen preparation, was grown in tryptose broth with thiamine (Difco Laboratories, Detroit, Mich) supplemented with 5% calf serum (inactivated at 56°C for 30 minutes). This strain of *Brucella* does not require added carbon dioxide for growth. One liter of medium in a 2-liter baffled Erlenmeyer flask was inoculated with about 5×10^9 viable *Brucella* cells and incubated at 37°C for 48 hours with continuous shaking. The growing cells were harvested near the end of the log phase, and viable counts were done in the usual manner on tryptose agar with thiamine and 5% inactivated calf serum.

Preparation of Bru-Pel.—The bacteria were harvested by centrifugation at 5000 rpm ($4100 \times g$), washed once by suspension in cold, sterile, distilled water, and recentrifuged. They were then suspended in cold water to give a Klett (filter No. 66) reading of 470–500, which represented a viable count of 1×10^{10} to 2×10^{11} bacteria per ml. (This and all subsequent operations were performed in sterilized equipment; sterile distilled water was used throughout.) The aqueous suspension was treated with diethyl ether by the methods of Badakhsh and Foster (7) and Ribi et al (8). Two volumes of cold diethyl ether was added to 1 volume of the cold aqueous suspension of *Brucella* and the mixture was agitated for 15 minutes with a model E Vibro-

mixer (Chemapec Inc, Woodbury, NY). The mixture was then transferred to a separatory funnel and allowed to stand overnight at 25°C. The ether layer was discarded and the aqueous phase containing the extracted bacteria was retained. Nitrogen was bubbled through the aqueous cell suspension until the odor of ether was undetectable. The extracted cells were pelleted by centrifugation, washed with distilled water, and finally recovered by lyophilization of the aqueous suspension to yield Bru-Pel. Bru-Pel was relatively nontoxic; the LD50 was in the range of 7–15 mg/25-g mouse. The aqueous material that was discarded after removal of the bacteria contained a significant amount of crude *Brucella* lipopolysaccharide.

Animals.—Female Swiss-Webster mice, weighing 25–30 g, were obtained from Taconic Farms, Germantown, NY.

Other methods.—Detailed methods have been published elsewhere for production and assay of interferon (1), for challenge with SFV (1), and for preparation of and challenge with S180 cells (6).

RESULTS

Bru-Pel as an Antiviral Agent

Bru-Pel induces circulating interferon in mice of the so-called virus type, ie, type I interferon, which peaks at 6–8 hours. In this regard *Brucella* is unique among gram-negative organisms, all of which induce an endotoxin-type of interferon response which peaks at 2 hours. Since these aspects of interferon production have been amply documented elsewhere (1), no further details are presented. It should be noted that effective antiviral preparations of *Brucella* are also always effective interferon inducers. However, our data and those of others suggest that the antiviral action of *Brucella* preparations is not necessarily linked to interferon.

The test system used in table 1 is SFV infection of mice. Doses of Bru-Pel as low as 8 μg/mouse, given 24 hours prior to challenge, protect mice against SFV. Table 2 shows that the prophylactic action of larger doses of Bru-Pel (2000 μg) is still effective ≥ 7 days after inoculation. These results gave us our first evidence that the antiviral activity of Bru-Pel may be associated not only with the production of interferon, but also with a general increase in the level of nonspecific resistance in animals.

We have reported (9) that extraction of Bru-Pel with a mixture of chloroform-methanol (C-M) (2:1, vol/vol) yielded an insoluble residue (extracted cells) and a C-M extract. Neither extracted cells nor C-M extract alone induced interferon or afforded protection against SFV infection in mice. Full interferon-inducing and protective activity was restored when extracted cells were recombined with the C-M extract. C-M extract from heat-killed *Escherichia coli* was also effective in restoring activity to extracted *Brucella* cells. These results suggest that the antiviral properties of Bru-Pel are constituted of a C-M-extractable component and an unextractable component that is unique to *B. abortus*.

TABLE 1.—Protection of mice against SFV by Bru-Pel given 24 hrs prior to virus infection

Treatment*	SFV (200 PFU)* Mortality (total treated = 15 mice)	%	*P* value
Controls, phosphate-buffered saline at – 24 hrs	13	86	
Bru-Pel at – 24 hrs (μg)			
500	1	6	< 0.001
125	2	13	< 0.001
31	0	0	< 0.001
8	5	33	< 0.005
2	8	53	< 0.1†
0.5	9	60	< 0.2†

* In 0.5 ml ip.
† Not statistically significant.

Antitumor Activity of Bru-Pel

Our initial experiments on the antitumor activity of Bru-Pel (6) established that Bru-Pel inhibited ascites formation and death in mice challenged ip with S180 tumor cells. In this experiment, all 15 mice injected with 10^4 cells of S180 developed ascites; 14 mice died, with a mean day of death of 23.6 ± 4.7. Single doses of Bru-Pel ranging from 2000 to 125 μg given 1 day before injection of S180 cells significantly inhibited ascites formation and death. Lower doses were not tested.

TABLE 2.—Influence of interval between Bru-Pel injection and challenge with SFV on antiviral effect

Time of injection of Bru-Pel (2000 μg/mouse)	SFV challenge (ip) No. of dead mice/No. inoculated*	% mortality	Significance level (*P*)
– 1 day	1/15	6.6	< 0.001
– 2 days	0/15	0	< 0.001
– 4 days	2/15	13.3	< 0.001
– 7 days	0/15	0	< 0.001
– 14 days	9/15	60	Not significant

* Control values were 11 of 15 mice (73.3% mortality).

TABLE 3.—Protection of mice against S180 ascites tumor: effect of time of injection of Bru-Pel (*from* ref 6)

Bru-Pel (2000 μg ip)	S180 (10^6 cells ip on Day 0)			
	Ascites*		Mortality*	
	No. of mice with ascites/ No. inoculated†	%	No. of dead mice/No. inoculated†	%
Phosphate-buffered saline control	10/10	100	10/10‡	100
− 7 days	3/9	33	3/9	33
− 4 days	1/10	10	1/10	10
− 1 day	2/10	20	1/10	10
0 day	1/10	10	0/10	0
+ 1 day	2/10	20	1/10	10
+ 3 days	2/10	20	2/10	20
+ 7 days	2/9	22	2/9	22

*Observation period, 50 days.
†− 7 days, $P < 0.005$; − 4 to + 7 days, $P < 0.001$.
‡Mean day of death, 25.5 ± 3.94.

It was of interest to determine the effect of varying the time of injection of Bru-Pel to see if this material could exert a curative as well as a prophylactic effect. Since 2000 μg of Bru-Pel protected completely against 10^4 cells of S180 given 1 day later, this dose was used to determine the time period during which Bru-Pel was effective. Table 3 shows that there is little difference in protection when Bru-Pel was given as early as 7 days before or as late as 7 days after injection of the tumor cells. Bru-Pel did not protect significantly when injected 14 days before or 10 days after challenge with S180 cells.

The influence of the number of S180 cells injected on protection by Bru-Pel was determined by varying the dose of tumor cells and holding the Bru-Pel dose constant (6). An inoculum of as few as 10^3 cells of S180 killed 80% of mice by 50 days, with a mean day of death of 29 ± 4.8; with increased doses of tumor cells, the mean day of death decreased. A single Bru-Pel injection (2000 μg) given 1 day before challenge with doses of S180 cells ranging from 10^3 to 10^6 cells significantly reduced mortality and prolonged survival times. When 10^7 cells of S180 were used as challenge, ascites and death were not prevented by a single dose of Bru-Pel; however, the survival time was increased by 25% ($P < 0.001$).

Although Bru-Pel was most effective when administered ip, it displayed some activity when injected by another route. Iv injection of Bru-Pel (2000 μg) into mice 1 day before tumor cell challenge did not significantly prevent ascites or death. However, the mean day of death was delayed significantly: 20.3 ± 3.09 for untreated controls and 27.5 ± 9.23 for treated animals ($P < 0.01$).

DISCUSSION

The experimental data and observations we have presented suggest that in addition to inducing interferon, Bru-Pel is a potent nonspecific inducer of resistance in mice; presumably, it acts by stimulation of cells of the reticuloendothelial system. These effects are similar to those which have been observed with other stimulants of nonspecific resistance, ie, *Corynebacterium parvum* and bacillus Calmette-Guérin preparations, which are being used for the experimental therapy of human cancer. The ability of Bru-Pel to protect animals, even when given after challenge with tumor cells, makes Bru-Pel a likely candidate for further investigation as an antitumor agent.

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Comparative Effects of *Corynebacterium parvum, Brucella abortus* Extract, Bacillus Calmette-Guérin, Glucan, Levamisole, and Tilorone With or Without Cyclophosphamide on Tumor Growth, Macrophage Production, and Macrophage Cytotoxicity in a Murine Mammary Tumor Model [1]

Bernard Fisher* and Mark Gebhardt [2,3]

SUMMARY

In this laboratory, it has been repeatedly demonstrated (using a murine mammary tumor model) that the combination of cyclophosphamide (CY) and *Corynebacterium parvum* (CP) is more effective than either agent alone in the control of tumor growth. This paper presents information obtained in our model comparing findings on the effects of CP with a *Brucella abortus* extract (Bru-Pel; BP) and glucan (GL) on tumor growth. In addition, the influence of those agents as well as bacillus Calmette-Guérin, tilorone, and levamisole on bone marrow macrophage colony production and cytotoxicity is presented. None of the nonspecific stimulating agents (NSSAs) inhibited tumor growth when administered systemically without CY, confirming our previous contention that such immunotherapy alone is likely to be an ineffectual form of treatment. Whereas tumor regression was observed following intratumor CP, neither GL nor BP had such an effect. When used with CY, neither BP nor GL administered ip or intratumorally inhibited tumor growth as effectively as did CP and CY. Inhibition of the growth of a distant tumor as well as the treated tumor occurred following intratumor CP and CY but not following intratumor BP and CY. All of the microbial NSSAs increased macrophage colony production to varying degrees in both normal and tumor-bearing mice. In the latter mice, CP had the most prolonged effect. Levamisole and tilorone failed to increase colony production in normal mice while in tumor-bearing mice the effect was inversely proportional to the amount of agent administered. To some extent, the stimulation of colony production by the NSSAs paralleled the degree of tumor inhibition observed when those agents were combined with CY. The cytotoxicity of cultured macrophages could not be related to tumor growth inhibition.

[Cancer Treat Rep 62:1919–1930, 1978]

Repeated observations in a murine system used by us have shown that when *Corynebacterium parvum* (CP) is used in combination with cyclophosphamide (CY), the resultant tumor growth inhibition is greater than when either agent is used alone (1–4). To enhance the effectiveness of the combination, variation in administration (dosage, timing, and sequence) of both components was investigated (3), and different chemotherapeutic agents were substituted for or added to CY (2). Similarly, several other nonspecific immunomodulators (bacillus Calmette-Guérin [BCG], tilorone hydrochloride, levamisole, methanol-soluble fraction of *Mycobacterium butyricum* [MSF-MB], and *Brucella abortus* [Bru-Pel]) have been substituted for or added to the CP in an attempt to improve the immunotherapy com-

[1]Supported by contract N01-CB-64044 (Division of Cancer Biology and Diagnosis) and Public Health Service grants CA-12102 and CA-14972 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, and by funds contributed by Joseph Katz of Pittsburgh, Pa.

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ponent of the combination (5,6). Information has also been obtained from the model on the effect of CP on bone marrow macrophage production in normal (7) and tumor-bearing (8) mice, on lymph node (9) and macrophage (10) cytotoxicity, on the metabolism of CY (11), and on the recovery of bone marrow during treatment with CY (10). This report presents information obtained in our model comparing findings on the effects of CP with Bru-Pel (BP) and glucan (GL) on tumor growth. In addition, the influence of those agents as well as BCG, tilorone, and levamisole on bone marrow macrophage colony production and cytotoxicity is presented.

MATERIALS AND METHODS

Mice.—Mice used in this study were inbred C3HeB/FeJ females, 8–12 weeks of age.

Tumor.—The tumor used was a spontaneous mammary carcinoma arising in a C3H/HeJ female and was maintained by transfer in C3HeB mice. In all experiments, tumors were transferred by inoculating 2×10^5 viable tumor cells in 0.1 ml of M199 sc into the left hind leg distal to the popliteal node; the tumors that arose were designated as "primary." A "distant" tumor was produced by injecting the right hind leg in the same manner at the same time with either 2×10^5 or 5×10^4 tumor cells. Prior to the beginning of treatment, two diameters of the tumor at right angles were measured using a vernier caliper and were averaged. Mice were assigned to various groups in the experiment so that each contained primary tumors of equivalent size. Control mice received 0.9% NaCl solution by the appropriate route at the time when treatments were given to test animals. Since averages ± SD of tumor growth for a group of mice fail to portray adequately the variability of response to treatment, growth curves for individual animals in an experiment are also presented.

Cyclophosphamide.—This agent was prepared in distilled water so that the desired amount, 60 mg/kg of body weight, was contained in 0.01 ml/g of body weight. It was administered ip every 7 days.

Nonspecific stimulating agents (NSSAs).—The agents used in these studies, their dosage, route of administration, and source are listed in table 1.

Combined therapy.—When CP, GL, or BP was used in combination with CY, it was given 4 days after each CY administration. When intratumor immunotherapy resulted in complete regression of a tumor, subsequent inoculations were made sc in the region of the regressed tumor.

Bone marrow macrophage assay.—A modification of the method of Bradley and Metcalf (12) used by us in previous studies and described in detail elsewhere (13) was employed. No colony-stimulating factor was utilized. The marrow cell suspension was counted and diluted to contain 1×10^6 cells/ml. Then, 1.5 ml of the cell suspension was added to 13.5 ml of medium 1066 containing 1.8% methylcellulose and 15% horse serum. One ml of the resulting suspension (containing 1×10^5 cells) was plated in each of ten tissue culture dishes (35 × 10 mm; Falcon Plastics Co, Oxnard, Calif). Plates were incubated at 37°C in a 10% CO_2 atmosphere with 100% humidity for 7 days. Identification marks on the plates were replaced by a code to ensure objectivity of the colony counts. Only groups of ≥ 25 cells arranged in a colony configuration were counted.

TABLE 1.—Immunopotentiators used

Agent	Dose and schedule	Source
CP, CN6134	1.4 mg q7days ip or intratumorally	Dr. J. K. Whisnant, Burroughs-Wellcome Co, Research Triangle Park, NC
Mycobacterium bovis strain BCG	1.0 mg equivalent wet weight q7days ip	Research Foundation, Chicago, Ill
GL *Saccharomyces cerevisiae*	4.2 mg q7days ip 0.45 mg q7days iv or intratumorally	Dr. N. R. Di Luzio, Tulane University, New Orleans, La
BP *Brucella abortus*	1.4 mg q7days ip, iv, or intratumorally	Dr. J. S. Youngner, University of Pittsburgh, Pa
Levamisole 1-Tetramisole	5 mg/kg q7days ip 5 mg/kg q2days ip 2 mg/kg/day orally	Dr. E. W. Cantrell, Lederle Laboratories, Pearl River, NY
Tilorone Tilorone hydrochloride	1 mg q7days ip 1 mg q1day ip	Dr. A. Richardson, Jr, Merrell-National Laboratories, Cincinnati, Ohio

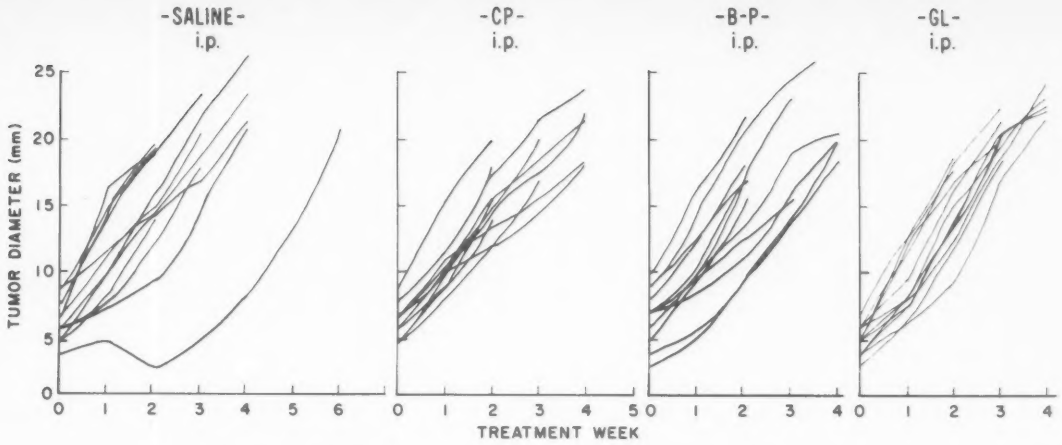

FIGURE 1.—Effect of systemic (ip) CP, BP, or GL on growth of a single tumor.

Bone marrow macrophage cytotoxicity assay.—To produce large numbers of macrophages for cytotoxicity testing, a colony-stimulating factor mouse L-cell (NCTC, clone 929)-conditioned medium was added to the culture medium. After 7 days' incubation the cells harvested were macrophages, with rare exceptions (14). The macrophages were isolated from the culture medium by washing four times with 0.9% NaCl solution and were resuspended in Earle's minimal essential medium with 30% fetal calf serum. The cytotoxicity of the macrophages was assayed by the method previously described for lymph node cells (15). The cells to be tested were added to wells in microtest plates which were covered by a monolayer of tumor cells. Each plate included wells with no added cells, macrophages from nontumorous mice, and the macrophages to be assayed. Plates were incubated for 48 hours, stained with crystal violet, and examined. The wells were scored on a scale of 0–5 according to the area free of tumor cells, 0 indicating complete coverage and 5 indicating complete absence of tumor cells. A sufficient number of plates were employed to provide 30 wells treated with each type of cell. The data are presented as the percentage of tumor cell destruction, which compares the amount of tumor remaining in the treated wells to that in the nontreated control wells.

RESULTS

Effect on Tumor Growth

Neither CP, BP, nor GL administered systemically (ip) affected growth of tumors in animals harboring a single tumor focus (fig 1). When the CP was inoculated intratumorally, 20% of tumors totally regressed, whereas intratumor administration of BP or GL produced no better effect than did ip administration (fig 2). When BP or GL was administered ip with CY, tumor growth inhibition was less than that obtained when CP was similarly given with CY. BP added to CY, however, resulted in slightly better inhibition than that obtained with CY alone (fig 3). The relative effectiveness of treatment using either CP or BP is well-demonstrated by average tumor growth (fig 4). After 8 weeks of CY and CP (ip) therapy, three of 12 tumors completely regressed and two others were smaller than when treatment was begun. No complete regressions resulted from CY, BP, or GL therapy and no tumors were smaller than when therapy was begun (fig 3).

A striking inhibition of tumor growth was achieved with intratumor CP and systemic CY (fig 5). Intratumor BP and ip CY had a greater effect than did ip BP with CY (fig 6), but the results with intratumor BP were not as good as those with intratumor CP. With both agents total tumor regression occurred; more tumor regression followed the use of CP. Administration of iv GL with CY produced results that were a little better than those with CY alone, whereas intratumor GL and CY inhibited the growth of tumors in a third of the animals (fig 5). Neither CP, BP, nor GL administered alone improved survival of tumor-bearers. When combined with CY, enhanced survival resulted only with CP and BP (fig 7).

The addition of BP or GL to CP and CY treatment resulted in several total regressions, but the overall

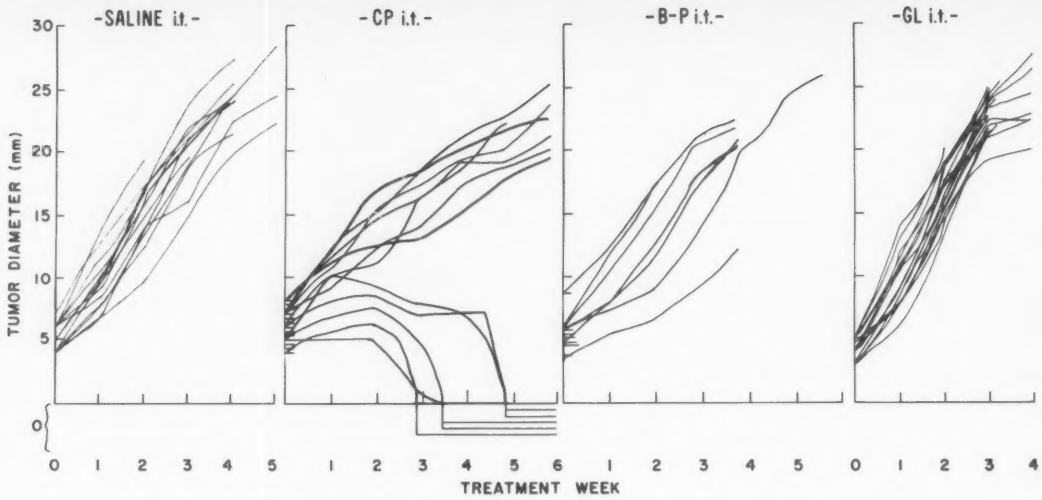

FIGURE 2.—Effect of intratumor CP, BP, or GL on growth of a single tumor.

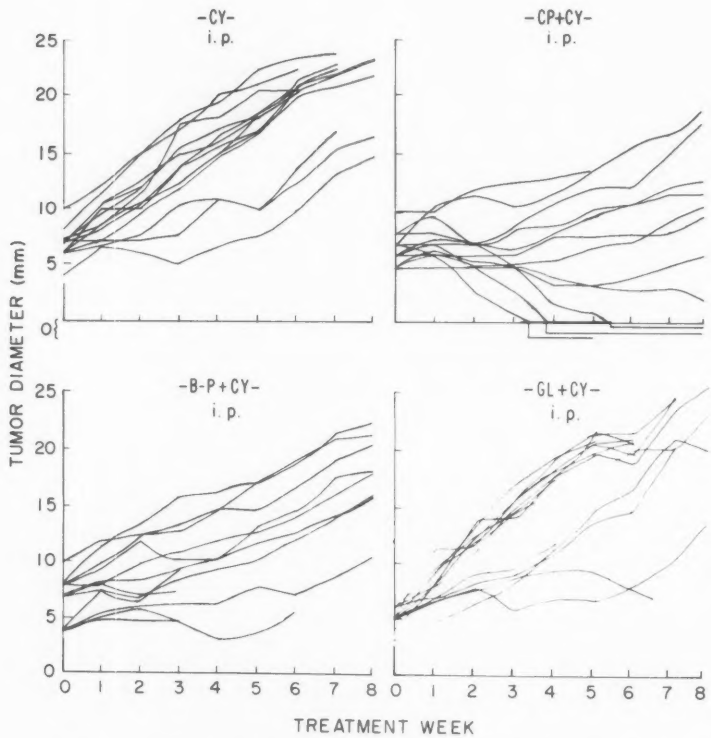

FIGURE 3.—Effect of systemic (ip) CP, BP, or GL with CY on growth of a single tumor.

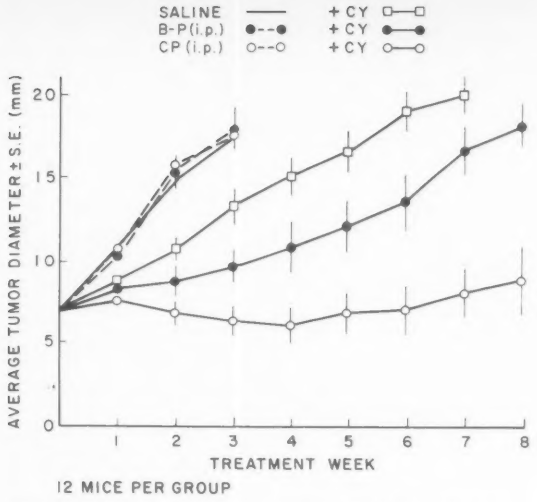

FIGURE 4.—Comparison of CY + CP with CY + BP—effect on tumor growth.

effect of the two-drug combination was not significantly improved (fig 8). The addition of BP resulted in the death of 41% of the animals in contrast to no mortality when CP and CY were used without the second NSSA. Similar mortality (58%) occurred with the addition of GL.

When mice bearing two tumors (a primary on the left hind leg measuring 3–5 mm and a distant tumor focus on the right hind leg measuring 1–3 mm) were treated with ip CY and CP given either ip or into the primary (intratumor), both the growth of the primary tumor and the growth of the distant tumor focus were more effectively inhibited by the intratumor CP (fig 9). When BP was used instead of CP in mice having two tumor foci of equal size (3–5 mm), the growth of the treated (primary) tumor was more effectively inhibited by the use of intratumor rather than ip BP. Growth of the distant tumor focus was only slightly affected when either of the two routes was used.

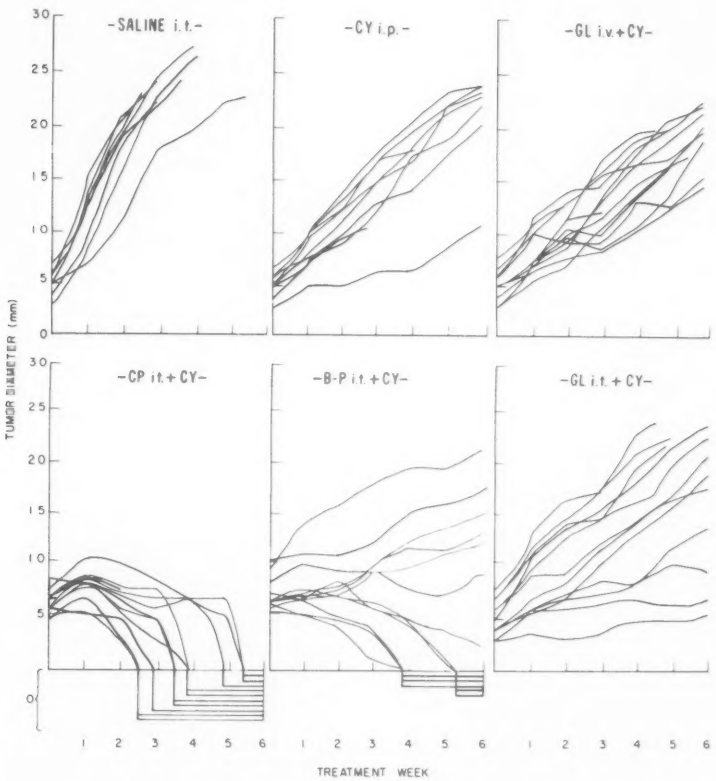

FIGURE 5.—Effect of intratumor CP, BP, or GL with CY on growth of a single tumor.

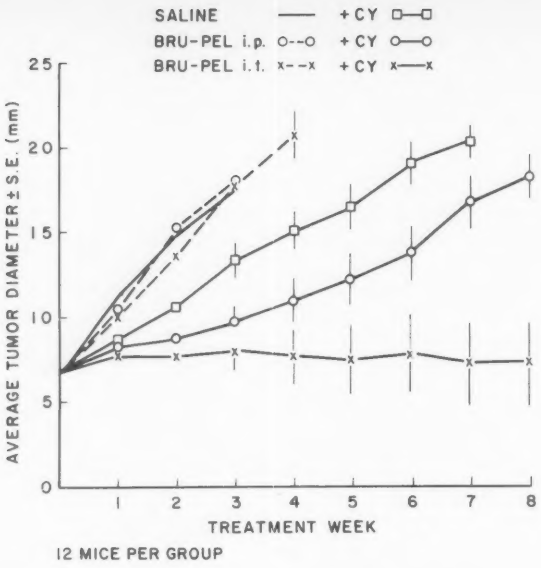

FIGURE 6.—Comparison of local to systemic BP on tumor growth.

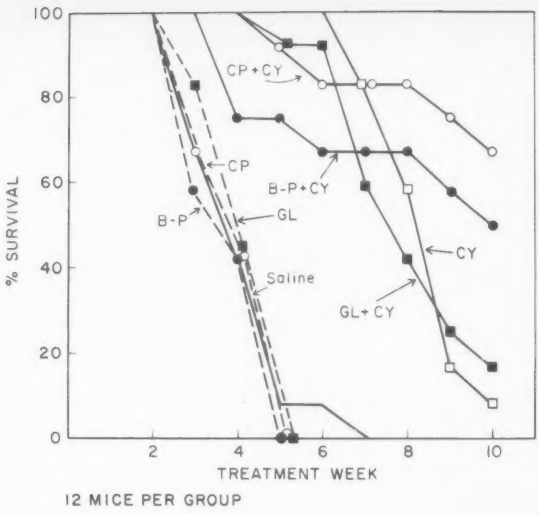

FIGURE 7.—Systemic CP, BP, or GL with or without CY—effect on survival.

Effect on Macrophage Colony Production

The effect of microbial NSSA administration on macrophage colony production was first evaluated in normal mice (fig 10). A single dose of the four agents, CP (1.4 mg ip), GL (4.2 mg ip or 0.45 mg iv), BP (1.4 mg ip), or BCG (1 mg ip), produced essentially the same results. A significant change in colony production over that observed in saline controls was noted 1 day after inoculation; on the second day all agents produced an increase in colony production well over 100% of the control values. On the third and fourth days the stimulatory effects of CP, GL (iv), and BP were still evident. By Day 7, the values obtained with all agents were only slightly different from those observed in control mice. Increased colony production was more prolonged following iv GL administration than after its ip use. The nonmicrobial immunopotentiators, levamisole and tilorone, failed to produce the increases noted with the microbial NSSAs. Findings from tumor-bearing mice (fig 11) indicated that 3 days following CP or BP there was a 150% increase in macrophage colony production. By 7 days, whereas the CP effect persisted at about the same level, the BP effect was only slightly greater than that in control mice. GL, either ip or iv, produced much less of an effect on colony production than did either CP or BP. Results with BCG were intermediate to those obtained with BP or GL and were inferior to findings with CP. A single dose of levamisole or tilorone caused an increase in colony production which was less than that resulting from CP. Increasing the amount administered of either levamisole or tilorone inversely affected the findings.

Effect on Macrophage Cytotoxicity

In vitro cytotoxicity of macrophages cultured from bone marrow cells (CMAs) of tumor-bearing mice receiving the NSSAs indicated that CP or BCG failed to augment cytotoxicity, whereas CMAs from animals 3 and 7 days following a single dose of either BP or GL demonstrated greater cytotoxicity than did CMAs from controls (fig 12). Levamisole, whether administered once or three times a week, resulted in a decrease in CMA cytotoxicity. The decrease was less pronounced when levamisole was administered continuously in the drinking water. The effect of tilorone was related to the frequency of administration. A single weekly injection had little effect on CMA cytotoxicity, whereas daily administration resulted in a marked depression of CMA cytotoxicity which persisted for ≥ 7 days after the last administration.

DISCUSSION

We have previously reported that despite a diversity of dosages and schedules of administration,

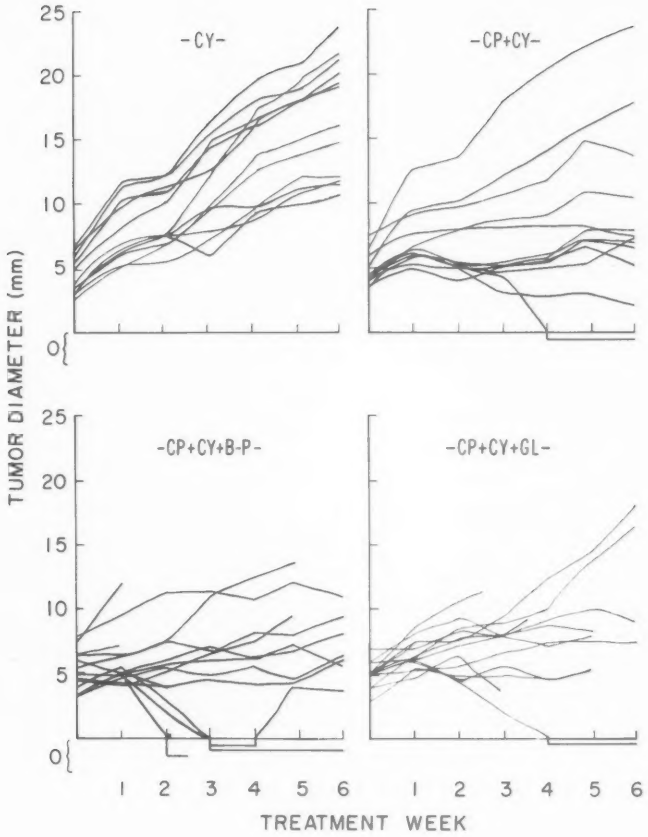

FIGURE 8.—Addition of systemic (ip) BP or GL to CY and ip CP—effect on tumor growth.

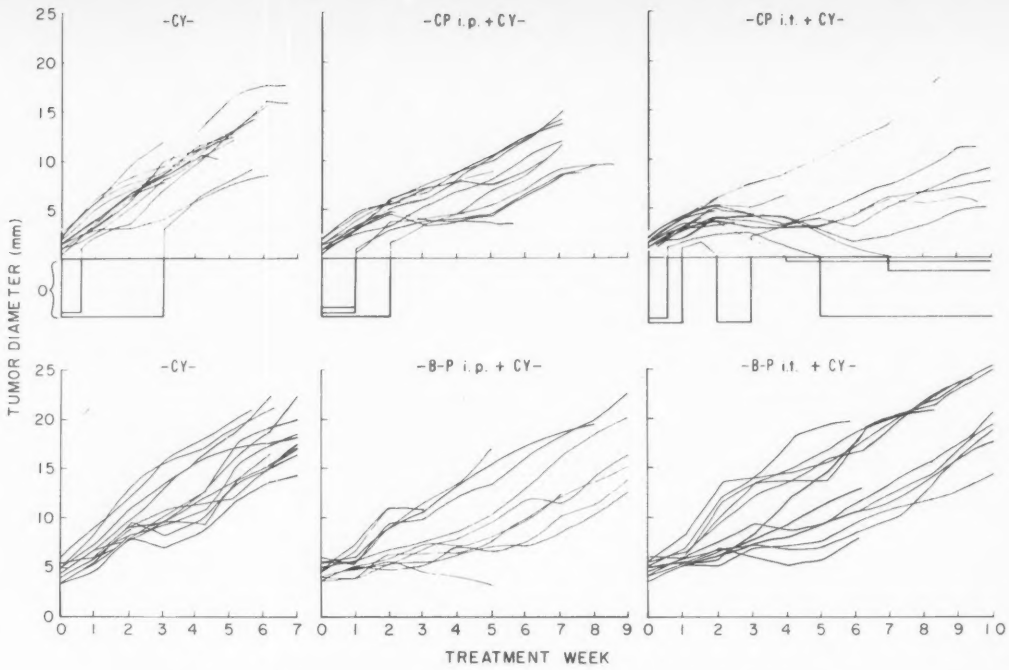

FIGURE 9.—Effect of intratumor CP or BP with CY on growth of a distant tumor.

none of a series of immunomodulators was as effective or more effective than CP when used alone or substituted for the CP in combination with CY (5,6). The immunopotentiators used (tilorone, levamisole, MSF-MB, and BCG) were those which have been used as antitumor agents in animal systems, have been clinically studied, and have been presumed to have variable mechanisms of action. The present studies indicate that two additional agents which more recently became available to us, BP and GL, were likewise less effective in the control of tumor growth. The former (BP) is a potent interferon-inducing antivirus agent which has significantly protected animals harboring an ascitic sarcoma (16,17). An equivalent dose of CP was significantly less effective in the same tumor model (17). GL, a β-1,3-glucosidic polyglucose derived from yeast cell wall, has been demonstrated to be a profound reticuloendothelial system-stimulating agent and to successfully inhibit growth and dissemination of allogeneic leukemia cells (18,19). Neither of the agents when administered systemically without chemotherapy inhibited tumor growth. This is in agreement with our previous observations using other immunomodulators in a similar manner and confirms our contention that such immunotherapy by itself is likely to be an ineffectual form of treatment (5,6). The failure of intratumor BP or GL to restrain tumor growth in this model is similar to findings employing BCG, but differs from those obtained with intratumor CP. Total tumor regressions have occurred as a result of the use of the latter agent. As reported for tilorone, levamisole, MSF-MB, and BCG (5,6), when employed with CY, neither BP nor GL administered ip or intratumorally inhibited tumor growth as effectively as did CP in combination with CY. Of significance was the observation that the local administration of CP in conjunction with CY, but not BP, inhibited growth of a distant tumor as well as the locally treated tumor, suggesting that treatment of a primary tumor with NSSA may be advantageous for the control of the growth of metastases.

The macrophage is considered to be an important effector cell in host defense against tumor. Certain NSSAs (CP, GL, and BCG) have been observed to increase the production of bone marrow macrophage precursors in normal (7,20–23) or tumor-bearing (8,21) animals. Consequently, it seemed worthwhile to compare the stimulation of macrophage production by the various NSSAs so as to determine whether differences between agents in that regard could account for the dissimilar effects on tumor growth resulting from their use. We have deter-

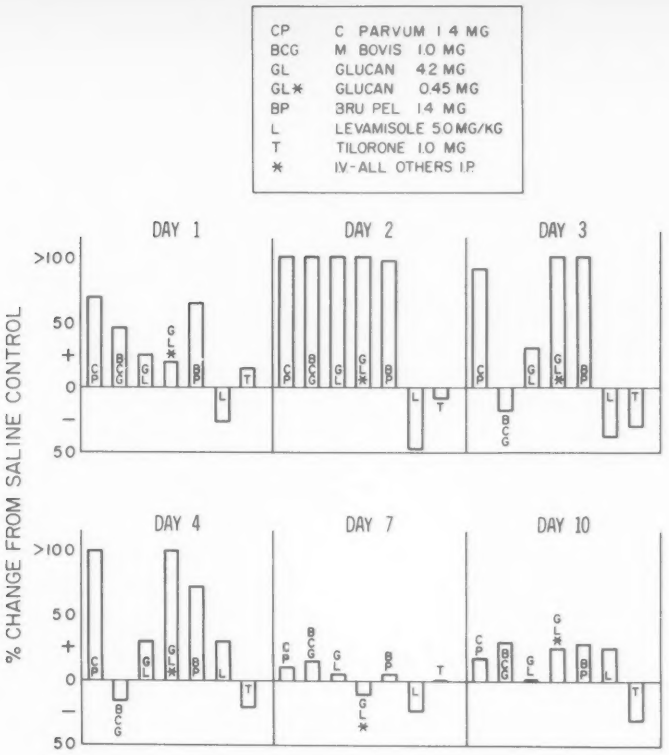

FIGURE 10.—Effect of immunopotentiators on macrophage colony production (colonies/10^5 bone marrow cells)—normal mice.

mined that in vitro CMAs of tumor-bearers are specifically cytotoxic to an immunizing tumor and that the cytotoxicity is derived from the antecedent colony-forming and/or stem cell in bone marrow (24,25). Consequently, the effect of the various NSSAs on such cytotoxicity was determined to ascertain whether quantitative differences in that parameter could account for the divergent effects on tumor growth. It was of interest that all four of the microbial NSSAs (CP, BCG, GL, and BP) increased macrophage colony production to a similar degree in normal mice whereas the nonmicrobial immunopotentiators, levamisole and tilorone, failed to do so. The findings with GL are similar to those reported by others who noted an increase in colony-forming cells in the spleen, bone marrow, and peritoneum following two doses of ip GL (20). Those findings with CP and/or BCG are in keeping with our prior findings (7,21) and those more recently observed by others (22,23). The present finding from tumor-bearing mice indicating that macrophage colony production is more pronounced following administration of CP than following the use of other agents extends our previous observations (8). While BP produced an initial increase comparable to that of CP, the augmentation was less sustained. Colony production following administration of levamisole and tilorone seemed to be inversely related to the amount of those NSSAs given.

Findings indicated that the cytotoxicity of cultured macrophages was not augmented by CP or BCG. In contrast, BP and GL increased cytotoxicity whereas levamisole and tilorone depressed that function. The more tilorone that was administered the greater the depression. Whereas the cytotoxicity of CMAs observed as a result of tumor growth with or without administration of CP was highly specific to the immunizing tumor (25), there is some evidence from preliminary investigations that the augmented cytotoxicity of such cells resulting from BP and GL administration may have a nonspecific component.

It is of interest that while all of the microbial NSSAs increased macrophage colony production to varying degrees when administered systemically, none of the agents were demonstrated to be effec-

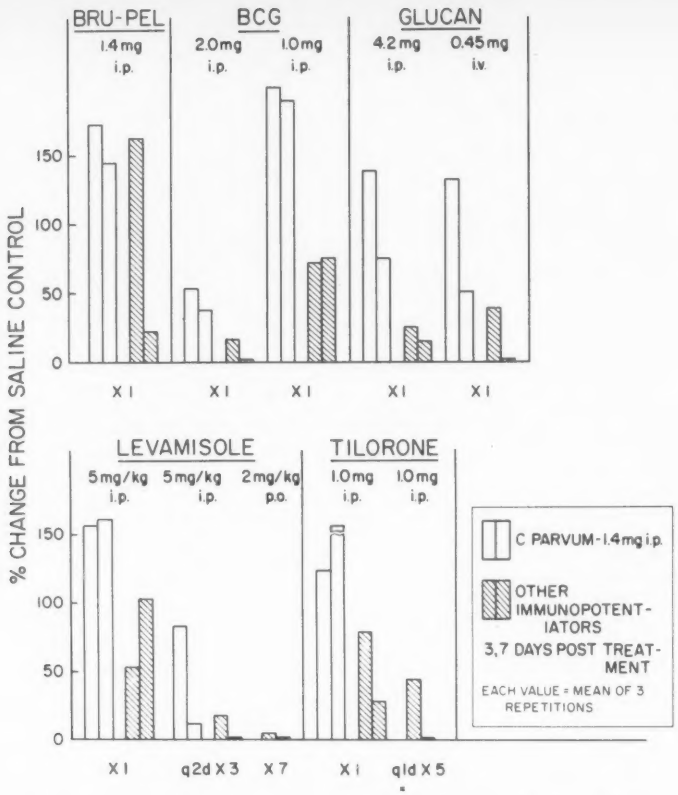

FIGURE 11.—Effect of immunopotentiators on macrophage colony production (colonies/10^5 bone marrow cells)—tumor mice.

tive in inhibiting tumor growth if given without chemotherapy. On the other hand, when they were employed with CY the inhibition of tumor growth to some degree paralleled the stimulation of colony production by the NSSAs. Similarly, the cytotoxicity of CMAs could not be related to the antitumor effect of the various NSSAs employed alone. While CP was observed not to increase CMA cytotoxicity, we have reported that CP, when administered with CY, resulted in the presence of a larger number of colony-forming cells in bone marrow at the nadir and after recovery from CY, thus indicating an increase in the absolute number of cytotoxic cells (10). The cytotoxicity of the CMAs varied following use of the other NSSAs, but the absolute number of CMAs generated during treatment with CY and those NSSAs is not known. Consequently, while the findings suggest that there is no correlation between CMA cytotoxicity and tumor-growth inhibition, until information is available concerning the depression and recovery of bone marrow colony-forming cells when the other agents are used with CY, no conclusions regarding the correlation of cytotoxicity and tumor growth can be made.

There remains the possibility that since a multiplicity of doses and schedules was not tested relative to the use of each agent, they may not have been employed under optimal circumstances in this model. Although the effectiveness of CP in combination with CY has been shown to be optimal when a specific dose and chemoimmunotherapy interval were used, the value of this combination was still evident with the use of a wide variation of those parameters (3). Alternatively, if the findings obtained with the other agents, despite the variations employed, are related to their improper administration, then their worth as effective immunomodulators must be considered to be less than that of CP. If the factors controlling their successful use are so precise, only serendipity may elucidate them.

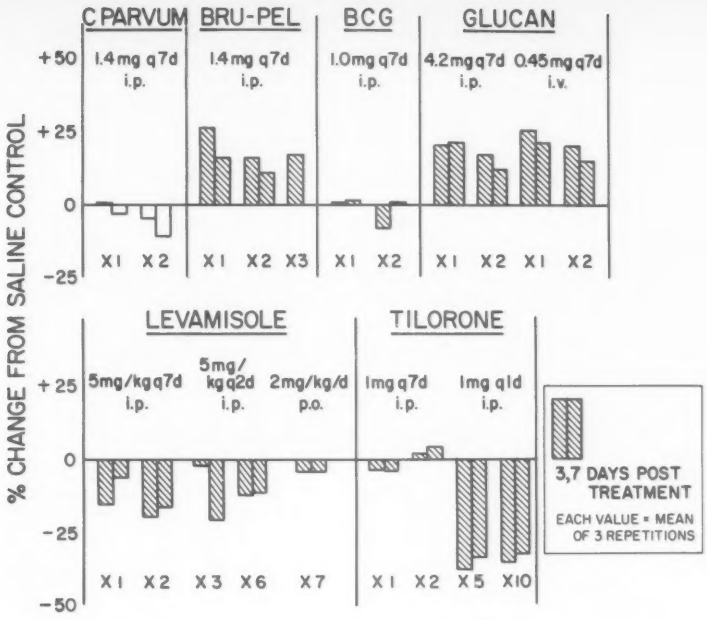

FIGURE 12.—Effect of immunopotentiators on cytotoxicity of cultured macrophages.

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Activation of Reticuloendothelial System Macrophages and Enhancement of Host Resistance to a Transplantable Osteogenic Sarcoma in Mice by an Extract of *Brucella abortus* [1]

Lowell A. Glasgow,[2,*] John L. Crane, Jr,[2] Charles J. Schleupner,[2] Earl R. Kern,[2] Julius S. Youngner,[3] and David S. Feingold[3]

SUMMARY

An aqueous-ether extract of *Brucella abortus*, Bru-Pel, enhanced resistance of mice to a transplantable osteogenic sarcoma (OGS). The results presented in this report suggest that Bru-Pel is an effective immunomodulator and that one mechanism through which it enhances host resistance is activation of phagocytic cells of the reticuloendothelial system. Peritoneal macrophages from mice inoculated with Bru-Pel 14 days previously were cytotoxic for OGS cells in vitro, limited the multiplication of vaccinia virus in cell cultures, and demonstrated increased chemiluminescence during phagocytosis. Furthermore, Bru-Pel enhanced host resistance to *Listeria monocytogenes*, in addition to viral infections and a transplantable tumor. These results support the hypothesis that Bru-Pel shares a number of characteristics with other recognized immunomodulating agents and suggest that further studies are warranted to better define the potential of Bru-Pel for immunotherapeutic regimens in man.

[Cancer Treat Rep 62:1931–1935, 1978]

A number of bacteria and bacterial products have the capacity to modulate host resistance. *Corynebacterium parvum* and bacillus Calmette-Guérin (BCG) are the most widely recognized immunomodulators and are currently being utilized in the treatment of malignancies in man (1,2). More recently, preparations of *Brucella abortus* have been shown to induce interferon production, protect against viral infections, and enhance resistance to both solid tumors and leukemia in experimental animals (3–6). Hoping to develop a preparation which would have fewer extraneous antigenic materials and fewer undesirable side effects, Youngner and co-workers (7,8) developed an aqueous-ether extracted preparation of *B. abortus*, Bru-Pel, which was a nontoxic interferon inducer and which protected animals against a lethal infection with Semliki Forest virus. Subsequently, in our own laboratory we confirmed the interferon-inducing capacity of Bru-Pel as well as its ability to modulate host resistance to viral infections in several murine experimental models (9). More pertinent to this symposium, however, is the evidence presented by Keleti and co-workers (10) that mice could be protected from death due to Sarcoma 180 when Bru-Pel was administered from 7 days before inoculation of the sarcoma cells to as late as 7 days after tumor cell injection.

The studies to be reported here demonstrate the capacity of Bru-Pel to enhance host resistance to the development of a transplantable osteogenic sarcoma (OGS) in mice and define some of the effects of this new immunomodulator on reticuloendothelial system function. The data further suggest that activation of macrophages is one mechanism through which Bru-Pel exerts both its antitumor and its antiviral activity.

[1]Supported in part by Public Health Service grants AI-06264 and AI-10217 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health, Education, and Welfare.

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TABLE 1.—Interferon titers in serum of adult mice injected with Bru-Pel or poly I:C (*from* ref 9)

Interferon inducer (μg/mouse)	Interferon titers (units/ml) after injection				
	3 hrs	6 hrs	9 hrs	12 hrs	24 hrs
Bru-Pel					
1000	3075	4475	2150	3000	75
500	1100	3850	1075	2325	50
100	400	3225	1050	500	50
Poly I:C					
100	3850	3750	5275	3400	150

MATERIALS AND METHODS

Mice.—C57BL/6J female mice, 6–10 weeks of age (Jackson Laboratories, Bar Harbor, Me), were used in all experiments.

Immunomodulators, interferon inducers, and viruses.—Bru-Pel was prepared as previously described (7) and was supplied as a lyophilized powder by J. Youngner. Each mouse received 1000 μg of Bru-Pel ip in 0.1 ml of phosphate-buffered saline (PBS). *C. parvum* was received from Burroughs Wellcome Co, Research Triangle Park, NC, as a killed vaccine containing 7 mg/ml wet weight of organisms. The vaccinia virus pool was prepared in mouse embryo fibroblast (MEF) cells and titered 2×10^6 plaque-forming units (PFU) per ml in MEF cells. The origin and preparation of the MS strain of herpes simplex virus type 2 (HSV-2) utilized in these studies have been described previously (11).

Cell cultures and media.—A continuous line of OGS cells was established from a primary tumor induced in C57BL/6 mice exposed to ^{239}plutonium. This line has remained tumorigenic upon ip or sc inoculation into C57BL/6J mice. Peritoneal cells (PC) were harvested from experimental and control mice as previously described (12).

Chemiluminescence procedure.—Chemiluminescence of macrophages during phagocytosis was carried out as previously described (13). Zymosan particles were incubated in pooled human sera, centrifuged, and resuspended in PBS to provide a stock suspension of opsonized zymosan. Similarly, a culture of a clinical isolate of *Candida albicans* was heat-killed, centrifuged, washed, resuspended, and incubated in pooled human sera, and was again centrifuged and resuspended in PBS to provide a preparation of opsonized *C. albicans*. PC were exposed to opsonized zymosan or *C. albicans* particles at a 1:100 ratio. Duplicate vials from each test group were counted for 1 minute at approximately 10-minute intervals for 2 hours in a Beckman LS 100C system in ambient light and temperature in the out-of-phase mode with the front photomultiplier disconnected.

Listeria monocytogenes *infection.*—A strain of *L. monocytogenes* was obtained from G. B. Mackaness (Trudeau Institute, Saranac Lake, NY). Mice were inoculated with serial twofold dilutions of a 10% spleen suspension obtained from animals dying from a lethal infection with *L. monocytogenes*. The size of the inoculum was adjusted using the optical density of the suspension in comparison with a reference standard suspension of *L. monocytogenes*. The number of organisms was quantitated by determination of colony counts.

RESULTS

As background for our consideration of Bru-Pel as an immunomodulator, the data establishing Bru-Pel as an interferon inducer are briefly reviewed. Pooled blood samples from six mice were obtained at 3, 6, 9, 12, and 24 hours after injections of 1000, 500, or 100 μg/mouse of Bru-Pel. The results of one representative experiment are summarized in table 1. Peak interferon titers of 3000–5000 units were observed 6 hours after injection of Bru-Pel. Relatively high levels persisted through 12 hours and declined to barely detectable levels by 24 hours after inoculation. These data clearly indicate that Bru-Pel induces a relatively high level of interferon in the serum of mice which persists over a 12–24-hour period.

Antiviral activity of Bru-Pel.—The capacity of Bru-Pel to modulate host resistance to a viral infection by mechanisms other than induction of interferon was demonstrated in the next series of experiments. Six-week-old mice were inoculated ip with HSV-2 either 1, 4, 7, 10, or 14 days after receiving 2000 μg of Bru-Pel. The results of one representative experiment are presented in table 2. Control animals that received PBS 14 days prior to infection with HSV-2 had a mortality rate of 87%. Protection was observed when Bru-Pel was inocu-

TABLE 2.—Nonspecific resistance to HSV-2 infection in 15 adult mice induced by Bru-Pel administered at various times prior to viral challenge (*from* ref 9)

Treatment* (days)	No. of deaths (%)	*P* value
PBS (– 14)	13 (87)	—
Bru-Pel (– 1)	4 (27)	< 0.01
Bru-Pel (– 4)	12 (80)	Not significant
Bru-Pel (– 7)	7 (47)	0.05
Bru-Pel (– 10)	5 (33)	< 0.01
Bru-Pel (– 14)	2 (13)	< 0.001

*Treatment with 2000 μg was initiated 1–14 days prior to infection with HSV-2.

TABLE 3.—Enhancement of resistance to OGS in C57BL/6J mice inoculated with Bru-Pel or *C. parvum*

Treatment (days)	No. of dead mice/ No. inoculated	% mortality	*P* value
Control	29/30	97	—
Bru-Pel (– 14)	11/30	36	< 0.001
Bru-Pel (0)	7/27	28	< 0.001
Bru-Pel (+ 3)	6/18	33	< 0.001
Bru-Pel (+ 7)	13/18	84	0.04
C. parvum (– 14)	15/28	54	0.001
C. parvum (0)	7/24	29	< 0.001

lated 1 day prior to virus, presumably through the induction of interferon. In contrast, those animals that received Bru-Pel 4 days prior to virus challenge were not protected. We have interpreted this to be due to the loss of antiviral effect of the interferon prior to virus challenge. In the remainder of the experimental groups, an increasing level of protection was demonstrated in animals receiving Bru-Pel 7, 10, and 14 days prior to HSV-2. These results strongly suggested that Bru-Pel is an effective modulator of host resistance and confirm the initial report (8) that Bru-Pel has antiviral activity.

Antitumor activity of Bru-Pel.—We next extended these studies to determine the capacity of Bru-Pel to modulate host resistance to a transplantable tumor. Groups of animals were inoculated with 1×10^5 OGS cells by the ip route either before or after treatment with 1000 μg of Bru-Pel. The results of two experiments are pooled and summarized in table 3. A significant degree of protection was observed when Bru-Pel was administered 14 days before, simultaneously with, or 3 days after inoculation of the OGS cells. Two groups of animals received *C. parvum* as an established immunomodulating agent. In these experiments, Bru-Pel effectively protected animals against the transplantable OGS cells with at least a similar degree of efficacy as *C. parvum*.

Mechanism of action of Bru-Pel.—The next series of experiments were designed to determine the effect of Bru-Pel on reticuloendothelial system function as one approach to defining the mechanism of action by which Bru-Pel modulates host resistance. To determine whether Bru-Pel activated peritoneal macrophages to be cytocidal for tumor cells, PC were harvested from syngeneic C57BL/6J mice 14 days after 1000 μg of Bru-Pel was administered by the ip route. A drop of the PC suspension was placed on the surface of each well of a disposable tissue culture plate, non-adherent cells were washed off, and the adherent cell population was overlayed with OGS or MEF cells. The number of PC was adjusted to provide a target cell ratio of 40:1, 20:1, 10:1, and 5:1. The results of one representative experiment are summarized in table 4. PC harvested from animals inoculated with Bru-Pel were cytocidal for OGS cells in vitro as evidenced by up to 90% destruction of the OGS cell monolayer in comparison with the lack of cytotoxic effect of PC from control mice. It should be noted that in our experimental system, the PC activated by Bru-Pel were also cytocidal for MEF cells as well as for the tumor cells and thus did not demonstrate specificity in their activity against tumor cells.

We previously described an in vitro assay system for demonstrating antiviral activity of macrophages in vitro by their capacity to limit the progression of a viral infection in monolayer cell cultures (14). PC from animals exposed to Bru-Pel 14 days previously or from control animals were added to cultures of MEF cells which had been infected with vaccinia virus. Samples of supernatant were harvested and assayed for virus. The data presented in figure 1 demonstrate that PC harvested from animals inoculated with Bru-Pel or *C. parvum* have the capacity to reduce viral multiplication in a susceptible culture of cells as evidenced by a decreased virus yield in the supernatant fluid in the cultures.

Another accepted manifestation of increased reticuloendothelial system activity is enhancement of host resistance to *L. monocytogenes*. In the next series of experiments, we determined whether Bru-Pel could alter host resistance to *L. monocytogenes* in vivo. Mice were inoculated with 1000 μg of Bru-Pel and then challenged with twofold serial dilutions of *L. monocytogenes* 14 days later. The final mortality of Bru-Pel-treated animals compared with control mice that received PBS is summarized in table 5. A striking degree of protection was observed in the two Bru-Pel-treated groups of animals that received a 100% lethal inoculum of *L. monocytogenes*. These data extend the immunomodulating

TABLE 4.—Cytocidal effect of macrophages from mice inoculated with Bru-Pel

	% destruction of cell monolayer								
		Bru-Pel				Control			
Cell	Control	40:1*	20:1	10:1	5:1	40:1	20:1	10:1	5:1
OGS	< 10%	> 90%	75%	40%	< 10%	< 10%	< 10%	< 10%	< 10%
MEF	< 10%	> 90%	75%	40%	< 10%	< 10%	< 10%	< 10%	< 10%

* Macrophage:target cell ratio.

capacity of Bru-Pel to the enhancement of host resistance to a bacterial infection.

One reflection of the metabolic processes stimulated after particle ingestion by phagocytic cells is the emission of light or chemiluminescence. We have adapted the technique utilized with polymorphonuclear neutrophils to murine peritoneal cells and demonstrated that chemiluminescence is enhanced in parallel with other parameters of macrophage activation (13). PC harvested from animals previously inoculated with Bru-Pel or from control animals were exposed to opsonized zymosan or *C. albicans* particles and the chemiluminescence emitted from the cells was determined. The results presented in figure 2 demonstrate a striking in-

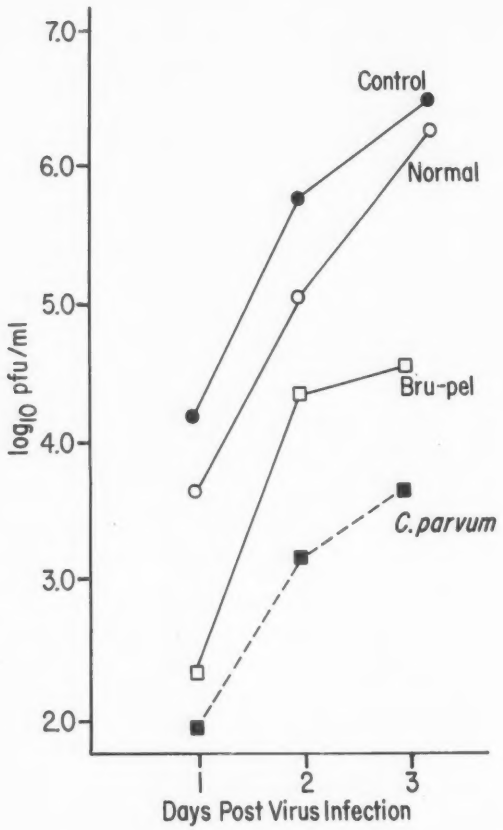

FIGURE 1.—Suppression of vaccinia virus replication in MEF cultures by peritoneal cells from mice inoculated with Bru-Pel or *C. parvum*.

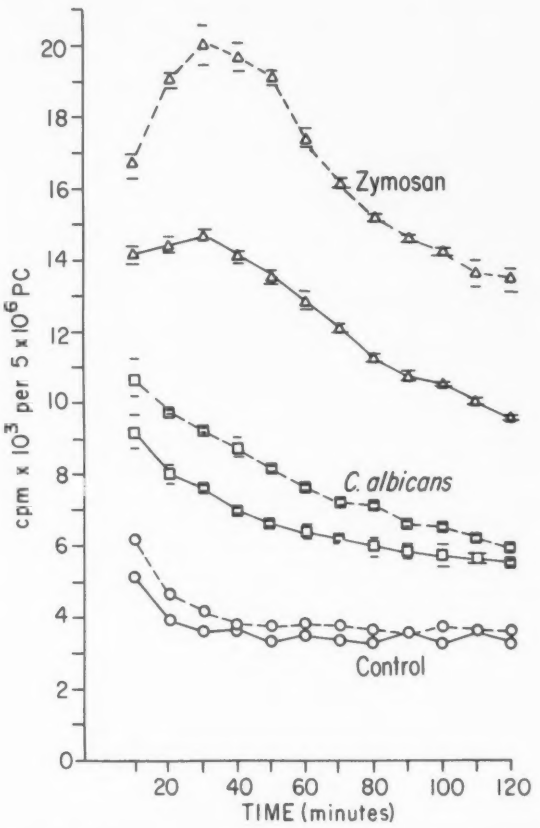

FIGURE 2.—Emittance of chemiluminescence (cpm) by peritoneal macrophages obtained from animals inoculated with Bru-Pel (----) or PBS (———) during phagocytosis of zymosan particles or *C. albicans*.

TABLE 5.—Enhancement of resistance of mice treated with Bru-Pel to infection by *L. monocytogenes*

Treatment	*L. monocytogenes* (CFU)	% mortality	No. of dead mice/No. inoculated
PBS	1.1×10^6	100	10/10
PBS	5.6×10^5	100	10/10
PBS	2.8×10^5	10	1/10
PBS	1.4×10^5	0	0/10
Bru-Pel	1.1×10^6	0	0/10
Bru-Pel	5.6×10^5	0	0/10
Bru-Pel	2.8×10^5	0	0/10
Bru-Pel	1.4×10^5	10	1/10

crease in chemiluminescence emitted from PC obtained from animals inoculated with Bru-Pel during phagocytosis of zymosan particles. A lesser difference was observed with *C. albicans* particles although the chemiluminescence emitted again appears greater for the PC activated by Bru-Pel.

DISCUSSION

Immunomodulators are currently being used in the treatment of human malignancies. Although *C. parvum* and BCG are the most widely utilized modulators of host resistance, other agents are recognized to have similar effects. The results presented in this report demonstrate that a nonviable insoluble residue of Bru-Pel shares many of the characteristics of other recognized immunomodulators. When inoculated 7–14 days prior to challenge, Bru-Pel strikingly enhances host resistance to viral infections, transplantable tumor cells, and a bacterial infection with *L. monocytogenes*. These studies support the concept that Bru-Pel may have potential as an effective immunomodulating agent, and suggest that further investigation of this substance is warranted.

The second series of studies reported here were directed toward elucidating the mechanism of action of Bru-Pel. Known immunomodulating agents are associated with activation of macrophages, including increased phagocytic activity and development of the capacity to be cytotoxic for tumor cells. The results of our experiments indicate that Bru-Pel activates macrophages, as evidenced both by the capacity of PC exposed to Bru-Pel to limit the progression of a viral infection in a cell culture system and to be cytocidal for OGS cells in vitro. Another measure of phagocytic cell function is the capacity to emit chemiluminescence during phagocytosis. Although this technique has been primarily utilized in studies with polymorphonuclear cells, it may also be associated with mononuclear phagocytes. The data presented indicate that Bru-Pel is an effective stimulant in increasing chemiluminescence in peritoneal macrophages during phagocytosis of zymosan particles.

In summary, Bru-Pel shares with other immunomodulators the capacity to enhance host resistance to challenge with viruses, transplantable tumors, and bacteria. The results presented support the hypothesis that at least one mechanism through which Bru-Pel modulates host resistance in these experimental models is through activation of reticuloendothelial cell function.

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Macrophage Activation and Antitumor Activity of a *Brucella abortus* Ether Extract, Bru-Pel

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SUMMARY

Bru-Pel and *Brucella abortus* lipopolysaccharide (LPS) were tested for both macrophage activation and antitumor activity in an artificial metastasis model. Resting macrophages were rendered nonspecifically tumoricidal for MBL-2 lymphoblastic leukemia target cells by exposure to Bru-Pel at ≥ 1 ng/ml of culture medium. *B. abortus* LPS failed to activate macrophages in vitro at all concentrations tested. Ip treatment of homozygous nude mice with Bru-Pel induced cytotoxic macrophages, indicating that Bru-Pel activated macrophages through a thymic-independent process. An artificial metastasis model was developed where single-cell suspensions of Madison 109 lung carcinoma were inoculated iv into syngeneic BALB/c mice. Bru-Pel, but not *B. abortus* LPS, strikingly inhibited tumor-colony formation in the lungs. Although Bru-Pel contains endotoxin, the data demonstrate that endotoxin is apparently not the active component by which Bru-Pel activates macrophages and enhances host resistance to cancer.

[Cancer Treat Rep 62:1937–1941, 1978]

Several bacteria or bacterial components have been observed to be potent stimulants of the macrophage system. Macrophages have been activated (rendered nonspecifically tumoricidal) in vivo by well-defined substances such as lipid A of gram-negative bacteria (1,2) and peptidoglycan (or mucopeptide) of bacterial cell walls (3,4). Alexander and Evans (1) demonstrated that lipid A of endotoxin acted directly on macrophages without lymphocyte involvement. In addition, chronic infection with *Mycobacterium bovis*, strain bacillus Calmette-Guérin, or treatment with killed *Corynebacterium parvum* produced activated macrophages as a result of lymphokine secretion from T lymphocytes (5,6).

Bru-Pel (an ether extract of *Brucella abortus*) has previously been shown to be a potent interferon inducer that enhances resistance to a variety of viral infections (7,8). In this report, we demonstrate that Bru-Pel directly activates macrophages and retards the development of artificially induced lung carcinoma metastases. Moreover, the active component of Bru-Pel appears to be separate from endotoxin.

MATERIALS AND METHODS

Mice.—Male BALB/c and $CD2F_1$ mice, 6–8 weeks old, were obtained from the Mammalian Genetics and Animal Production Section, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Homozygous nude mice were supplied by Charles River Breeding Laboratories, Wilmington, Mass. Mice were housed in plastic cages with air-filter bonnets and fed Purina laboratory chow and water ad libitum.

Drugs.—The methods for preparation of Bru-Pel, an aqueous ether extract of a living nonpathogenic strain of *B. abortus*, and *B. abortus* lipopolysaccharide (LPS) have previously been described (7). *Escherichia coli* LPS (0127:B8) and *Salmonella typhimurium* LPS were purchased from Difco Laboratories, Detroit, Mich. All drugs were made up in sterile Dulbecco's phosphate-buffered saline (PBS).

Target cells.—Established cell lines of MBL-2

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($H2^b$[C57BL/6]) murine leukemia and Madison 109 (M109) ($H2^d$[BALB/c]) murine alveolar carcinoma cells were maintained in RPMI-1640 medium supplemented with 20% heat-inactivated (56°C for 30 minutes) fetal calf serum (FCS), 100 μg/ml of gentamicin solution, 0.075% $NaHCO_3$, and 10 mM Hepes buffer (RPMI-FCS).

Peritoneal macrophages.—Noninduced peritoneal macrophages were harvested and purified by adherence as previously described (9). Representative preparations of purified adherent cells were stained with Giemsa stain; > 95% of the cells observed had morphologic characteristics of macrophages. Macrophages were kept in an ice bath prior to use to prevent adherence.

Test for activation of macrophages in vivo.—The ability of activated macrophages to diminish target cell proliferation was measured by the inhibition of DNA synthesis assay previously described (9). Briefly, target cells were trypsinized from exponentially growing cultures and resuspended at 5.0×10^4 cells/ml of RPMI-FCS, and 2-ml aliquots were placed in 30-mm tissue culture dishes. Purified peritoneal macrophages were adjusted to 1.0×10^6 cells/ml of RPMI-FCS, and 1 ml was added to the target cell cultures. DNA synthesis of the target cells was assessed after 20 hours of incubation at 37°C. Three cultures of each set of dishes as well as cultures consisting of tumor cells alone and macrophages alone were pulsed with 2.0 microcuries of tritiated thymidine (specific activity, 10 curies/mmol) for 2 hours at 37°C. At the end of the incubation, the cells were detached with trypsin, centrifuged at 600 × *g* for 10 minutes, and resuspended in 0.5 ml of Dulbecco's PBS. Four ml of chilled 10% trichloroacetic acid was added to each tube, and precipitation was allowed to continue for 30 minutes at 4°C. The resultant precipitate was collected on glass-fiber paper and washed with cold 10% TCA. The filters were air-dried and assayed for radioactivity using Aquasol solubilizer (New England Nuclear Corp, Boston, Mass). The percent inhibition of DNA synthesis was calculated by the following formula.

$$\% \text{ inhibition of DNA synthesis} = \frac{cpm_S - cpm_E}{cpm_S} \times 100,$$

where cpm_S = mean counts per minute in cultures containing effector cells from normal control mice and cpm_E = mean counts per minute in cultures containing test effector cells.

Test for direct macrophage activation in vitro.—The method used for measuring the ability of drugs to produce growth inhibitory macrophages in vitro has previously been described (10,11). Approximately 4×10^5 peritoneal macrophages contained in 1 ml of RPMI-FCS were seeded in 16-mm wells on Tissue Culture Cluster Plates (Costar, Cambridge, Mass). After 2 hours of incubation at 37°C in a 5% CO_2-in-air atmosphere, nonadherent cells were removed by three cycles of aspiration with RPMI-1640 medium. The adherent cells (macrophages) were then overlaid with 4×10^4 MBL-2 cells contained in 1.0 ml of RPMI-FCS. Drugs were added in the desired concentrations in an additional 1.0 ml of RPMI-FCS. Toxicity controls consisting of MBL-2 cells alone in the presence of the various drugs were also included in each experiment. Drugs were kept in the culture medium for the duration of the incubation. All cultures were maintained in a humidified, 5% CO_2-in-air incubator at 37°C, and viable leukemia cells were counted daily with a hemocytometer. Percent growth inhibition of MBL-2 cells due to macrophage-drug interaction was calculated by comparison to MBL-2 cells grown in the presence of normal macrophages alone.

Inhibition of artificially induced lung metastases.—M109 cells grown in vitro were harvested during their exponential growth phase by gentle trypsinization, were washed twice, and were resuspended in serum-free RPMI-1640. The number of single viable cells was determined and adjusted to 1×10^5 cells/ml of medium. Tumor cells were injected iv in normal BALB/c mice. Inoculum volume per mouse was 0.2 ml (2×10^4 cells). Drugs were given ip to randomized groups of mice 5 days prior to tumor cell inoculation. Controls received 0.2 ml of Dulbecco's PBS. Five mice from each group were killed on Days 14 and 20, and the number of pulmonary metastases was determined by India ink inflation by the technique of Wexler (12).

RESULTS

Direct macrophage activation in vitro by bacterial agents.—Bru-Pel was compared to various LPS preparations for the ability to transform normal resting macrophages into cytotoxic effector cells in vitro (table 1). Bru-Pel activated macrophages to inhibit MBL-2 leukemia cell growth with optimal activity at 10 μg/ml, although significant activity remained at concentrations as low as 1 ng/ml. LPS preparations derived from *E. coli* and *S. typhimurium* showed kinetics similar to those of Bru-Pel. In contrast, LPS from *B. abortus* did not have any effect on resting macrophages at any of the concentrations tested. None of these preparations had a direct inhibitory effect on MBL-2 target cells in the absence of macrophages.

Macrophage activation in vivo by Bru-Pel.—We

TABLE 1.—Ability of bacterial agents to activate murine peritoneal macrophages in vitro

Bacterial agent	Growth inhibition* at 48 hrs of— 100 μg/ml	10 μg/ml	1 μg/ml	100 ng/ml	10 ng/ml	1 ng/ml
Bru-Pel	++	++	++	+	+	+
B. abortus LPS	NT†	–	–	–	–	–
E. coli LPS	++	++	++	++	+	–
S. typhimurium LPS	++	++	++	+	–	–

*– = Growth inhibition of MBL-2 target cells < 25%; + = between 25% and 50%; ++ = > 50% of control value.
†NT = not tested.

studied the effect of ip Bru-Pel treatment on macrophage reactivity from BALB/c and athymic nude (nu/nu) mice. Macrophages from both strains of mice potently arrested DNA synthesis of M109 lung carcinoma cells when harvested 6 days after Bru-Pel treatment (fig 1). Macrophage activation by Bru-Pel was thymus-independent and was not dose-dependent over a range of 1–100 mg/kg.

Effect of Bru-Pel on artificially induced pulmonary metastases.—Pyran copolymer (NSC-46015) has previously been shown to retard the development of experimental M109 lung metastases (13). To determine whether Bru-Pel was similarly effective in potentiating surveillance against M109 pulmonary metastases, Bru-Pel or 0.9% NaCl solution was administered ip 5 days prior to iv tumor inoculation. Mice were killed on Days 14 and 20, and their lungs were inflated with India ink in order to visualize tumor nodules. Bru-Pel strikingly reduced the number of pulmonary lesions in two separate experiments (table 2). In contrast to multiple tumors in the lungs of placebo-treated mice, a few lungs (four of ten) from Bru-Pel-treated mice remained tumor-free on Day 20. *B. abortus* LPS was without effect on experimental metastasis development.

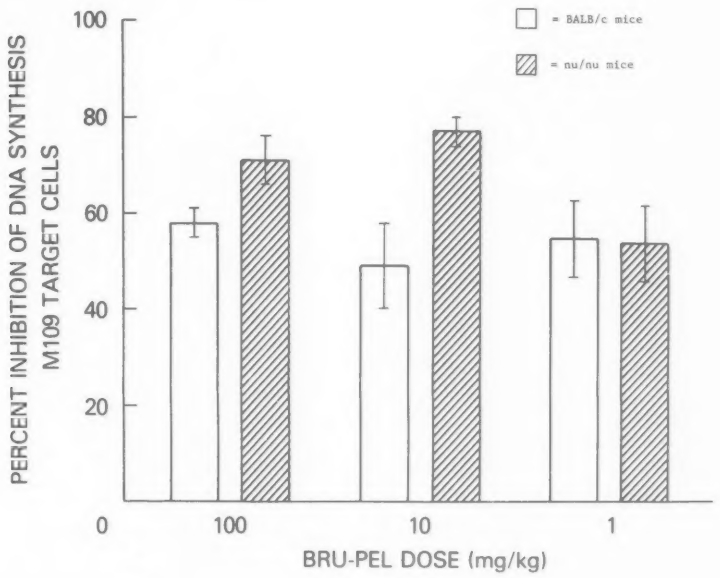

FIGURE 1.—Effect of Bru-Pel on the cytostatic activity of purified macrophages from BALB/c and nu/nu mice for M109 target cells. Bru-Pel was administered ip 6 days prior to harvesting the peritoneal macrophages. The bars = the mean ± SE derived from 3 determinations.

TABLE 2.—Inhibition of artificially induced lung metastases of M109 lung carcinoma by Bru-Pel

Drug treatment*	Mean No. of lesions ± SE/lung† (Day 14)	No. of mice with lung tumors/ total No. of mice	Mean No. of lesions ± SE/lung† (Day 20)	No. of mice with lung tumors/total No. of mice
Experiment 1				
Control	4.0 ± 0.4	5/5	117.0 ± 54.3	5/5
Bru-Pel (Day – 5)	0.0 ± 0.0	0/5	2.8 ± 0.9	4/5
Experiment 2				
Control	9.4 ± 1.8	5/5	> 250	5/5
Bru-Pel (Day – 5)	0.2 ± 0.2	1/5	0.6 ± 0.4	2/5
B. abortus LPS (Day – 5)	5.0 ± 2.0	4/5	> 250	5/5

* Bru-Pel and *B. abortus* LPS were administered ip at 100 and 2 mg/kg respectively.
† 2×10^4 M109 cells were injected iv on Day 0. Lung lesions were identified by inflation with India ink.

DISCUSSION

Many investigators have suggested that cytotoxic macrophages play an important if not crucial role in host defense against neoplasia. Hibbs (14) advanced the theory that nonspecifically activated macrophages in the host provide an effective antitumor surveillance system by differentiating transformed from normal cells in a somatic population and by selectively killing these transformed cells by a secretion of lysosomal enzymes of macrophage origin into the cytoplasm of the susceptible cell (15). We have recently demonstrated that the ability of adjuvants to enhance host resistance against cancer correlated with the capacity to produce cytotoxic macrophages (16). Similarly, Fidler (17) presented evidence from an experimental metastasis model that the iv injection of specifically activated macrophages into mice significantly reduced the number of established pulmonary metastases. In addition, Bomford and Moreno (18) demonstrated a temporal correlation between the presence of cytostatic macrophages and lung metastasis inhibition after injection with *C. parvum* and a number of polysaccharides.

In this paper, we show that Bru-Pel activated macrophages by a direct mechanism independent of T-lymphocyte factors. In this regard, Bru-Pel was similar to a number of polyanions in its kinetics of macrophage activation in vitro and may result from an interferon induction mechanism (10,11). Although Bru-Pel contains endotoxin (as measured by the Limulus amebocyte assay), we show that LPS derived from *B. abortus* lacked effect on macrophage functional activity. Although Alexander and Evans (1) showed that lipid A of gram-negative bacterial endotoxin activates macrophages, it appears that the lipid A of enterobacteria is different from *Brucella* (19). This might explain the inability of *B. abortus* LPS to activate macrophages in vitro.

Pretreatment of mice with Bru-Pel, but not *B. abortus* LPS, potently inhibited lung metastasis formation. Preliminary histopathologic observations of the host response to tumor give further support to the role of the activated macrophage in tumor surveillance. Macrophage accumulations in the interstitium of the lungs from Bru-Pel-treated animals were observed to encircle the metastatic foci and completely arrest their development. Our current data support the concept that activated macrophages have a surveillance function in inhibiting or controlling metastatic cell growth. Moreover, the enhancement of macrophage numbers and activity in the lungs following systemic Bru-Pel treatment could provide an approach to possible treatment.

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Comparative Adjuvant Effects of Levamisole and *Brucella abortus* in Murine Leukemia

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SUMMARY

The aqueous ether-extracted residue of *Brucella abortus* (Bru Pel) was found to possess immunoadjuvant activity when used in conjunction with chemotherapy for lymphocytic leukemia. It was most effective when administered by the iv or ip route. Single treatment was as effective as multiple treatment. Bru Pel treatment alone demonstrated a tumor-retarding effect against L1210 leukemia and the M109 alveolar carcinoma. Reduction of tumor burden by chemotherapy appeared to be a prerequisite for Bru Pel to exert its beneficial effect. The immunoadjuvant effect demonstrated by Bru Pel is related to its ability to stimulate cellular components of the immune system.

[Cancer Treat Rep 62:1943–1947, 1978]

In recent years, the application of nonspecific immune stimulators, when used alone or combined with other forms of therapy to augment host immunity, suggests that immunologic control measures may prove to be valuable adjuncts to the control of neoplasia. Bacillus Calmette-Guérin (BCG) affords protection against tumors induced with chemicals (1) or viral-transplantable cells (2). It has also been reported to be effective in controlling human leukemias (3) and malignant melanoma (4).

The use of chemicals known to stimulate cellular or humoral immune responses has not been investigated to a great extent to determine the effect of the chemicals when used in conjunction with chemotherapy for neoplasia. Levamisole has recently demonstrated immunoadjuvant activity when used in conjunction with chemotherapy for two different murine leukemias (5,6). The beneficial effect of levamisole and other agents when used as immunoadjuvants together with other treatment modalities for animal and human tumors has been reported (7,8).

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Bru Pel (an aqueous ether-extracted residue of *Brucella abortus*, strain 456) has been reported to be a potent interferon inducer that enhances resistance to a variety of viral infections (9,10). The antitumor effect reported for Bru Pel (11) appears to be mediated through a mechanism other than interferon. The objective of the present study was to determine whether Bru Pel, when used as an adjuvant to chemotherapy, would produce a beneficial effect in the treatment of a murine leukemia.

MATERIALS AND METHODS

Tumor

A Moloney lymphoid leukemia line, LSTRA, originally induced in BALB/c (H-2^d) mice by the Moloney murine leukemia virus, has been maintained and routinely passaged in our laboratory as a transplantable tumor line for several generations in (BALB/c × DBA/2)F_1 (H-2^d) (CD2F_1) mice. The ascitic tumor is serially transplanted ip at weekly intervals.

The M109 (H-2^d) (BALB/c) murine alveolar carcinoma tumor cell line has been maintained and routinely passaged in our laboratory as a transplantable tumor line for > 40 generations in BALB/c male mice. The tumor is serially transplanted sc at intervals of 3 weeks.

The L1210 (H-2^d) (DBA/2) murine leukemia tumor cell line has been maintained and routinely passaged in DBA male mice at weekly intervals by the ip route.

Mice

Adult $CD2F_1$ male mice, 6–8 weeks old, were obtained from the National Cancer Institute, Bethesda, Md. The animals were housed in plastic cages and fed Purina laboratory chow with water ad libitum.

Drugs

BCNU was kindly supplied by the National Cancer Institute. The alkylating agent was dissolved in a steroid-suspending vehicle and administered sc in a constant volume of 0.01 ml/g of body weight. Levamisole was kindly supplied by Dr. P. Janssen, Janssen Pharmaceutica, Beerse, Belgium. The chemical was dissolved in sterile 0.85% NaCl solution and administered ip in a constant volume of 0.01 ml/g of body weight. Bru Pel was supplied by Dr. J. S. Youngner, University of Pittsburgh, Pa. The aqueous ether-extracted residue was dissolved in sterile 0.85% NaCl solution and administered ip in a constant volume of 0.01 ml/g of body weight.

RESULTS

The response of LSTRA leukemia to chemotherapy and Bru Pel adjuvant treatment is shown in table 1. BCNU therapy was withheld until systemic leukemia was established (Day 7 after tumor inoculation). BCNU therapy alone was effective in prolonging the median survival time (MST) by 3 weeks over the control nontreated group, with 30% of the animals alive and tumor-free at 90 days when the experiments were terminated. The additional treatment with Bru Pel during the early drug-induced remission period (Day 12), 5 days after BCNU treatment, resulted in a more extended survival period and a larger number of tumor-free survivors. Multiple treatment with Bru Pel did not appear to be any more beneficial than single treatment.

Three groups of mice were treated with Bru Pel alone, by different routes, as comparative control groups to untreated and BCNU-treated mice to assess whether Bru Pel exerted a tumor-retarding effect. Bru Pel, when administered alone by the ip, iv, or intratumoral route, resulted in an increase of 41%, 25%, or 8% respectively.

The significant increases attained in both survival period (MST) and percentage of survivors demonstrate the synergistic effect exerted by combined BCNU and Bru Pel adjuvant treatment. Intratu-

TABLE 1.—Response of LSTRA leukemia to Bru Pel adjuvant

BCNU* (30 mg/kg)	Bru Pel			Survivors (%)	MST (days)
	mg/kg	Day	Route		
				0	12
+				30	33
+	4	12	ip	90	> 90
+	8	12		80	> 90
+	16	12		100	> 90
+	8	12, 19, 26		60	> 90
+	4	12	iv	80	> 90
+	8	12		60	> 90
+	16	12		100	> 90
+	8	12, 19, 26		60	> 90
+	4	12	Intratumoral	30	41
+	8	12		50	> 90
+	16	12		50	> 90
	16	12	ip	0	17
	16	12	iv	0	15
	16	12	Intratumoral	0	13

*Treatment 7 days after sc inoculation of 10^5 LSTRA cells.

TABLE 2.—Response of LSTRA leukemia to chemotherapy and levamisole and Bru Pel adjuvant

BCNU* (30 mg/kg)	Adjuvant (mg/kg)† Levamisole	Bru Pel	Route	Survival (%)	MST (days)
				0	12
+				30	32
+	5		ip	80	> 100
+	20			70	> 100
+		4		90	> 100
+		16		100	> 100
+	5		sc	90	> 100
+	20			70	> 100
+		4		30	42
+		16		50	> 100
+	5		iv	80	> 100
+	20			80	> 100
+		4		80	> 100
+		16		100	> 100
+	5		Oral	70	> 100
+	20			70	> 100
+		4		30	39
+		16		20	28

*Treatment 7 days after sc inoculation of 10^5 LSTRA cells.
†Treatment 5 days after BCNU therapy.

moral injection of Bru Pel did not appear to be as effective as the ip or iv route in increasing the percentage of survivors. The 16-mg/kg dose of Bru Pel appears to be the most effective dose of the three doses tested.

Table 2 contains the results of a study comparing the adjuvant effect of Bru Pel and levamisole, an agent previously reported to possess immunostimulatory effect. Similar, effective response rates were achieved with both Bru Pel and levamisole adjuvants when they were administered by the ip or iv route. No statistical differences were noted for the two doses tested of each adjuvant when administered by these two routes. Levamisole was more effective than Bru Pel when the sc route was employed. Although levamisole was effective when given orally, Bru Pel did not exhibit any adjuvant effect when given by this route. The responses attained by levamisole and Bru Pel when these agents were used in conjunction with chemotherapy show that both agents possess immunoadjuvant activity.

The increase in MST achieved by Bru Pel treatment alone in the LSTRA leukemia study (table 1) prompted us to investigate Bru Pel as a monotherapy in two other tumor systems, a leukemia (L1210) and a solid tumor (M109). The results in table 3 show that with decreasing concentrations of L1210 cell inoculum, progressive increases in MST over control values were attained. Although these increases in survival time resulting from Bru Pel treatment were consistent, they were not considered significant.

The results of a dose response study of Bru Pel in the M109 tumor system indicated that the dose of 100 mg/kg was most effective. The results of mono-

TABLE 3.—Effect of Bru Pel monotherapy on L1210 leukemia growth

Bru Pel* (mg/kg)	L1210 cell inoculum 10^3	10^4	10^5	10^6	10^7
Placebo	12†	11	9	8	7
	11–13‡	11–13	8–10	7–9	7–7
8	14	13	10	8	7
	13–15	12–13	9–10	8–9	7–7
16	16	14	11	8	7
	14–19	13–15	10–12	8–9	7–7

*L1210 tumor cell ip inoculation followed by Bru Pel ip injection on Day 0 within 3 hrs.
†MST (days) based on 20 animals.
‡Range of death (days).

therapy with Bru Pel at the 100-mg/kg dose are shown in table 4. Increases in lifespan were achieved when Bru Pel was administered by the ip or iv route. All other routes of Bru Pel treatment were found to be ineffective. The increases in lifespan correlated with the retardation of tumor growth observed in animals treated with Bru Pel by the ip or iv route (data not shown). It may be that multiple treatments with Bru Pel may have resulted in more significant effects in both the L1210 and M109 tumor systems; this possibility is presently being investigated. It is interesting to note that the best effects with Bru Pel were achieved when this agent was administered by the ip or iv route (tables 1–4).

Observations from many experiments in our laboratory indicate that the ability of the initial cytoreductive drug regimen to significantly reduce the tumor burden was essential in order for the immunoadjuvant to exert an additive beneficial effect. An evaluation of > 40 experiments was made on the tumor-reductive effect of cytoreductive therapy resulting from chemotherapy alone (BCNU, cyclophosphamide, methyl-CCNU, melphalan, or methotrexate), at the maximum tolerated antitumor dose of each drug and compared to the response achieved with the same drug plus adjuvant treatment (levamisole, Bru Pel, or DIVEMA). The data were divided into two categories: (*a*) reduction of the local tumor produced by sc inoculation of the LSTRA leukemia from the maximum tumor size of 12 mm to 0–4 or 5–10 mm; and (*b*) the percent of long-term remissions produced as the result of chemotherapy alone or combined chemotherapy plus adjuvant. The pooled data are presented in table 5. It became evident that only two drugs, BCNU and methyl-CCNU, were capable of producing a significant number of long-term remissions whether the initial tumor size was reduced to < 4 or 10 mm. In comparing the effectiveness of combined chemotherapy and immunoadjuvant, it is clear that in reducing tumor to a nadir of 0–4 mm, the immunoadjuvant provided a beneficial effect which resulted in a significantly higher percentage of long-term remissions. It was also evident that although a reduction of tumor to 5–10 mm occurred by chemotherapy alone, use of immunoadjuvant still produced a higher number of long-term remissions.

Another interesting response noted in this evaluation was that a higher percentage of animals that relapsed died with recurring local subcutaneous tumor and splenomegaly when they received chemotherapy alone, in contrast to animals relapsing that received chemotherapy plus adjuvant.

TABLE 4.—Effect of Bru Pel monotherapy on M109 adenocarcinoma when administered by different routes

Route of Bru Pel injection*	Survival (%)	MST (days)	Increase (%)
Placebo (ip)	0	30	—
Ip	0	40	33
Intralesional	0	34	13
Contralateral (sc)	0	30	0
Iv	0	41	36
Intradermal	0	32	6

*Mice inoculated with 1×10^6 cells sc and Bru Pel (100 mg/kg) administered 7 days later when tumors measured 5–8 mm.

DISCUSSION

Results of these studies demonstrate that Bru Pel, when used together with chemotherapy, exerts a beneficial additive effect resulting in a more prolonged survival and a higher percentage of ani-

TABLE 5.—Relationship of local tumor burden following chemotherapy and response to immunostimulator

Drug used for initial cytoreductive therapy of LSTRA leukemia	Long-term remissions (%)			
	Nadir tumor size (mm)			
	0–4		5–10	
	Chemotherapy alone	Chemotherapy + IS*	Chemotherapy alone	Chemotherapy + IS*
BCNU	30†	70‡	20	40
Cyclophosphamide	10	40	10	20
Methyl-CCNU	30	70	20	30
Melphalan	10	40	0	20
Methotrexate	0	10	0	0

*IS = immunostimulator: levamisole, Bru Pel, or DIVEMA.

†Most of the animals died with evidence of recurrent local tumor and splenomegaly.

‡Most of the animals died with splenomegaly.

mals cured of their leukemia. When used as a monotherapy, Bru Pel exerted a demonstrable, but not significant, effect on the L1210 leukemia. These results were not as significant as those reported for Bru Pel activity against the Sarcoma 180 tumor (11). A significant tumor-retarding effect was attained against a slow-growing alveolar carcinoma. A similar response was achieved by Bru Pel monotherapy of M109 tumor colony formation in the lungs (12).

The immunoadjuvant activity demonstrated by Bru Pel was similar to that obtained with levamisole, another reported immunostimulator. A major difference noted for these two agents was that effectiveness was dependent upon the route of administration. The most effective route for Bru Pel was ip or iv; in contrast, levamisole was active by all routes tested (ip, iv, sc, or oral). Bru Pel's immunoadjuvant activity was dependent upon the ability of the initial cytoreductive therapy to reduce the tumor burden, although Bru Pel alone did possess some tumor-retarding effect.

The mechanism through which Bru Pel may exert its effect was considered to be by stimulation of cells of the reticuloendothelial system (11). We have recently demonstrated that the ability of adjuvants to enhance host resistance against cancer correlated with the capacity to produce cytotoxic macrophages (13). In addition, Bru Pel has demonstrated adjuvant activity when combined with a poorly antigenic tumor (14). More recently, we have shown that Bru-Pel activates macrophages by a direct mechanism independent of T-lymphocyte factors and independent of any endotoxin contained in the Bru Pel preparation (12). The suppression of T and B cells resulting from tumor burden or cytoreductive chemotherapy was apparently reconstituted by the administration of Bru Pel (15).

The implication of these observations is that Bru Pel's immunoadjuvant activity is correlated strongly with its ability to activate, nonspecifically, tumoricidal macrophages and cause reconstitution of the T- and B-cell compartments depressed by cytoreductive therapy.

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Lymphocyte Stimulatory Effect of an Ether-Extracted Preparation of *Brucella abortus*

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SUMMARY

The effect of a *Brucella abortus* preparation (Bru Pel) on the T- and B-lymphocyte populations is described. Bru Pel treatment of tumor-bearing mice resulted in a slight reduction in tumor size, marked splenomegaly, and statistically significant reductions in the percentages of splenic T and B lymphocytes relative to untreated tumor controls. Bru Pel was also found to cause increases in the incorporation of ^{3}H-thymidine of phytohemagglutinin-sensitive splenic T lymphocytes and lipopolysaccharide-sensitive splenic B lymphocytes over ^{3}H-thymidine-incorporated values for untreated tumor controls.

[Cancer Treat Rep 62:1949–1954, 1978]

Ether extraction and subsequent centrifugation of *Brucella abortus* will yield a nonviable residue, Bru Pel. This preparation, when given ip either before or after challenge with Sarcoma 180 tumor cells, significantly inhibits both the development of ascites tumors and the death of animals (1). These antitumor effects are thought to be mediated by an increase in nonspecific resistance similar to that seen with other experimental antitumor agents (2).

In the present study, $CD2F_1$ mice were injected with Madison lung carcinoma 109. When all the injected mice had palpable tumors of at least 5 mm in diameter they were inoculated ip with either BCNU, Bru Pel, or BCNU and Bru Pel. We report here the effect of Bru Pel on the splenic T and B lymphocytes from these tumor-bearing mice.

MATERIALS AND METHODS

Animals.—$CD2F_1$ male mice, 6–8 weeks old, were supplied by the Mammalian Genetics and Animal Production Section, Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI) (Bethesda, Md). The animals were housed in plastic cages and fed Purina laboratory chow with water ad libitum. All animals weighed at least 23 g before use.

Drugs.—BCNU was kindly supplied by the Drug Synthesis and Chemistry Branch, DTP, DCT, NCI. BCNU was dissolved in hydroxypropyl cellulose (0.3% solution) and 30 mg/kg was administered ip. Bru Pel, an aqueous ether extract of a living nonpathogenic strain of *B. abortus* (3,4), was kindly supplied by Dr. J. S. Youngner of the University of Pittsburgh, Pa. It was dissolved in 0.9% sterile NaCl and 40 mg/kg was administered ip.

Tumor.—The Madison lung carcinoma 109 derived from a spontaneous neoplasm in a BALB/c mouse was kindly supplied by Dr. Ruth L. Geran, DTP, DCT, NCI, and has been maintained as a transplantable line in BALB/c mice. For these studies, 5.0×10^5 viable tumor cells were injected sc in the right inguinal region of $CD2F_1$ mice. The experimental animals attained palpable tumors of 5–7 mm in size by the 11th day after tumor inoculation. On the 12th day, animals which were to receive BCNU were distributed to the appropriate groups and were injected ip. The day of drug treatment was considered Day 0 and the in vitro assays were started 1 day later. The mice receiving Bru Pel (40 mg/kg) were injected ip on Day 5.

Lymphocyte preparation.—Lymphocytes were obtained from $CD2F_1$ mice by aseptic removal of the spleens. The spleens were minced in 10 ml of sterile RPMI-1640 medium supplemented with 10% heat-

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inactivated fetal bovine serum, 2 mM glutamine, and 1% penicillin-streptomycin in sterile 15 × 150-mm Petri dishes (Falcon Plastics, Oxnard, Calif). The dishes were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 1 hour in order to remove adherent macrophages. The nonadherent cells were collected and incubated at room temperature for approximately 5 minutes with 0.11 M ammonium chloride to deplete rbcs. The nonadherent, rbc-depleted cells, taken up in 4 ml of RPMI-1640, were layered over 5 ml of Ficoll-Hypaque separation medium containing 12 parts of 14% Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, NJ) and five parts of 32.8% Hypaque sodium (diatrizoate sodium, Sterling Winthrop Research Institute, Rensselaer, NY) (specific gravity = 1.09). The gradient was centrifuged at 300 × *g* for 30 minutes at room temperature. The interface, containing the separated lymphocytes, was collected, washed three times with RPMI-1640, checked for viability with trypan blue, and brought to a concentration of 3.3×10^6 viable cells/ml.

Antisera.—The production, titer, and specificity of the anti-T and anti-B sera used for indirect immunofluorescent studies have been described (5).

Immunofluorescence.—Indirect immunofluorescence was used to detect T- and B-cell surface markers utilizing the antisera mentioned above. Viable, nonadherent, rbc-depleted splenic lymphocytes (10^6) were incubated for 30 minutes with 300 μl of the appropriate dilution of the unlabeled antiserum (1:50 for the anti-B serum and 1:75 for the anti-T serum). The cells were washed three times in phosphate-buffered saline (PBS) containing 0.02% sodium azide and 10% heat-inactivated fetal bovine serum (Microbiological Associates, Bethesda, Md). After the third wash, the supernatant fluid was aspirated, and 300 μl of goat-anti-rabbit IgG at a dilution of 1:20 was added and incubation continued for 30 minutes at 4°C. The cells were again washed three times with PBS and were resuspended in 300 μl of the same buffer. Twenty μl of the cell suspension was placed on a glass slide and at least 100 cells (in triplicate) were counted in an American Optical microscope with a vertical fluorescent illuminator.

Statistical analyses.—Statistical treatment of the immunofluorescent data was performed using Student's t test (two-way).

Response to mitogens.—Viable, nonadherent, rbc-depleted lymphocytes (5.0×10^5) contained in 150 μl were added to each well of a Micro-test II Tissue Culture Plate (3040, Falcon Plastics). Lipopolysaccharide W (10 μg/ml) (Difco Laboratories, Detroit, Mich) or concanavalin A (Con A) (5 μg/ml) (Pharmacia Fine Chemicals) contained in 50 μl was also added. These concentrations were found to be optimal by wide-range titrations of the various mitogens. Cultures were incubated for 48 hours at 37°C in a 5% CO_2 and 95% humidified air atmosphere, and 0.5 microcurie of ^{3}H-methylthymidine (NET-027, specific activity, 6.7 curies/mmol; New England Nuclear Corp, Boston, Mass) in 50 μl was added to each well. The cultures were incubated for an additional 18–24 hours. The cells were washed with deionized water by use of a Mash II cell harvester (Microbiological Associates). All radioactive filters were counted in a liquid scintillation spectrometer (Packard Model 3385 Tri Carb, Downer's Grove, Ill) with Aquasol (New England Nuclear Corp) as the scintillation fluid. The results of triplicate cultures are expressed as mean cpm, that is, mean of triplicate cultures mitogen added minus mean of triplicate cultures no mitogen added.

RESULTS

Tumor development.—The growth of Madison lung carcinoma 109 in the various groups of treated mice is shown in table 1. BCNU treatment significantly reduced the size of the tumor as expected from this cytoreductive agent. Bru Pel treatment did not alter tumor size significantly (except for the Day-21 observation) although there appeared to be some retardation in tumor growth. The group of mice treated with the combination of BCNU and Bru Pel attained tumor sizes intermediate in value between the BCNU-alone and the Bru Pel-alone groups.

Spleen weight.—The cytoreductive activity of BCNU is also reflected by a decrease in spleen weight when compared to the tumored control (table 2). In tumored animals, splenomegaly appeared to be progressive. Spleen weights of tumored mice treated with Bru Pel were significantly greater for the Day-11–21 observations. These results are in agreement with previously reported observations of splenic weight increases resulting from Bru Pel treatment (4). Of particular interest were the intermediate splenic values obtained in the combined BCNU and Bru Pel-treated group. These values approximated those observed for the untreated tumored controls. It would appear that Bru Pel afforded a protective effect against the BCNU cytoreductive effect.

Immunofluorescence.—For all the indirect immunofluorescent studies, lymphocytes of the various groups of tumored mice were isolated from the spleen, purified by Ficoll-Hypaque gradient centrifugation, and reacted with either the anti-T or anti-B serum. The relative percentages of the T cells of

TABLE 1.—Tumor size of Madison lung carcinoma 109-bearing mice and tumor-bearing mice treated with BCNU and/or Bru Pel

Time (days)	Tumored control (mm)	Bru Pel alone (mm)	*P* value	BCNU + Bru Pel (mm)	*P* value	BCNU alone (mm)	*P* value
1	7.4 ± 0.8*					7.4 ± 0.6	> 0.10†
4	9.4 ± 0.8					8.0 ± 0.2	< 0.10
7	13.6 ± 0.7	12.4 ± 0.9	> 0.10	8.3 ± 0.7	< 0.001	6.5 ± 0.4	< 0.001
11	18.8 ± 0.9	17.6 ± 0.8	> 0.10	10.0 ± 0.4	< 0.001	9.6 ± 0.6	< 0.001
15	21.8 ± 1.1	19.2 ± 0.9	> 0.10	12.1 ± 0.6	< 0.001	11.2 ± 0.4	< 0.001
21	24.2 ± 0.6	20.4 ± 0.6	< 0.002	15.0 ± 0.7	< 0.001	19.5 ± 0.6	< 0.001

* Mean diameter of tumor ± SE; No. = 5–8.
† Probability is calculated vs tumored control, Student's t test.

TABLE 2.—Spleen weights of Madison lung carcinoma 109-bearing mice and tumor-bearing mice treated with BCNU and/or Bru Pel

Time (days)	Tumored control (g)	Bru Pel alone (g)	*P* value	BCNU + Bru Pel (g)	*P* value	BCNU alone (g)	*P* value
1	0.10 ± 0.00*					0.09 ± 0.00	< 0.02†
4	0.12 ± 0.01					0.05 ± 0.00	< 0.001
7	0.11 ± 0.01	0.09 ± 0.01	< 0.10	0.13 ± 0.04	< 0.10	0.06 ± 0.01	> 0.10
11	0.14 ± 0.01	0.21 ± 0.02	< 0.01	0.18 ± 0.02	< 0.10	0.09 ± 0.01	< 0.01
15	0.17 ± 0.02	0.29 ± 0.03	< 0.01	0.18 ± 0.01	> 0.10	0.11 ± 0.01	< 0.01
21	0.18 ± 0.02	0.31 ± 0.05	< 0.05	0.21 ± 0.02	> 0.10	0.14 ± 0.01	< 0.10

* Mean spleen weight ± SE; No. = 5–8.
† Probability is calculated vs tumored control, Student's t test.

the various groups of mice are shown in table 3. There is a definite decrease in the percentage of splenic T cells of the tumored control mice relative to the T cells detected for the untumored controls. The BCNU-treated mice had T-cell percentages which were not significantly different from those detected for the tumored controls. However, the Bru Pel-treated mice responded with T-cell percentages significantly higher than those detected for the tumored controls. The T-cell percentages of the mice treated with BCNU and Bru Pel were similar in value to those found for the BCNU-treated group. The B-cell percentages of the treated mice are shown in table 4. The untreated tumored controls resulted in a marked decrease of B cells as compared to the untumored controls. BCNU treatment resulted in a severe depletion of B cells. The Bru Pel-treated mice responded with significantly elevated B-cell percentages, whereas the group of mice treated with the combination of BCNU and Bru Pel had B-cell percentages higher than those detected for the BCNU-treated group, but lower than those detected for the Bru Pel group, indicating the possible protective effect of the Bru Pel treatment.

Lymphocyte proliferative response.—In order to augment the immunofluorescent data, mitogenic studies were carried out with splenic lymphocytes from the various groups of tumored mice using T- and B-cell mitogens. The mitogenic response of the splenic lymphocytes to Con A is shown in table 5. The Con A stimulation of the splenic lymphocytes from the tumored control mice is slightly lower than the values detected for the untumored mice. The lymphocytes from the BCNU-treated mice were not stimulated by Con A. Indeed, there is a 7.0–11.2-fold decrease in mean cpm relative to the tumored controls. Bru Pel-treated tumored mice incorporated less ^{3}H-thymidine (^{3}H-TdR) than did the tumored controls; however, the mean cpm values are 4.4–6.3-fold higher than the values detected for the BCNU-treated group. The BCNU and Bru Pel-treated mice resulted in mean cpm values intermediate between

TABLE 3.—% of T lymphocytes from the spleens of tumor-bearing mice and tumor-bearing mice treated with BCNU and/or Bru Pel

Time (days)	Tumored control (%)	Bru Pel alone (%)	*P* value	BCNU + Bru Pel (%)	*P* value	BCNU alone (%)	*P* value	Control (%)
1	33.0 ± 1.5*					29.0 ± 0.6	< 0.10†	23.0 ± 0.6
4	17.8 ± 1.8					19.3 ± 1.8	> 0.10	24.3 ± 0.9
11	23.7 ± 1.5	32.9 ± 0.9	< 0.01	24.7 ± 1.2	> 0.10	23.7 ± 1.5	> 0.10	24.7 ± 0.3
15	16.0 ± 0.6	30.7 ± 0.9	< 0.001	24.0 ± 0.6	< 0.001	23.0 ± 0.6	< 0.001	25.0 ± 0.6
21	14.3 ± 0.9	23.3 ± 0.7	< 0.01	22.3 ± 1.2	< 0.10	18.3 ± 1.2	> 0.10	25.0 ± 0.6

*Mean ± SE of at least 100 randomly enumerated lymphocytes; triplicate readings; No. = 5–8.
†Probability is calculated vs tumored control, Student's t test.

TABLE 4.—% of B lymphocytes from the spleens of tumor-bearing mice and tumor-bearing mice treated with BCNU and/or Bru Pel

Time (days)	Tumored control (%)	Bru Pel alone (%)	*P* value	BCNU + Bru Pel (%)	*P* value	BCNU alone (%)	*P* value	Control (%)
1	41.7 ± 1.2*					26.0 ± 1.0	< 0.001†	50.0 ± 0.6
4	40.0 ± 0.6					15.7 ± 0.3	< 0.001	47.3 ± 1.7
11	28.7 ± 0.9	50.0 ± 0.6	< 0.001	28.7 ± 0.9	> 0.10	15.0 ± 0.6	< 0.001	46.7 ± 0.9
15	26.7 ± 0.7	31.0 ± 0.6	< 0.01	26.0 ± 1.2	> 0.10	13.7 ± 0.3	< 0.001	48.7 ± 0.9
21	16.7 ± 1.8	28.3 ± 1.7	< 0.01	23.3 ± 1.8	< 0.10	13.3 ± 0.7	> 0.10	50.3 ± 0.3

*Mean ± SE of at least 100 randomly enumerated lymphocytes; triplicate readings; No. = 5–8.
†Probability is calculated vs tumored control, Student's t test.

the BCNU- and Bru Pel-treated groups, again indicating the possible protective effect of the Bru Pel treatment. The phytohemagglutinin (PHA) stimulation of the splenic lymphocytes from the tumored control mice is essentially the same as that detected for the untumored mice (table 6). Lymphocytes from the BCNU-treated mice not only failed to be stimulated by PHA, but resulted in negative mean cpm values on Days 15 and 31, indicating a severe depletion of PHA-sensitive splenic lymphocytes by BCNU. On the other hand, splenic lymphocytes from the Bru Pel-treated mice resulted in 1.2–1.6-fold increases in mean cpm values over the tumored control group. Similar to the results obtained with Con A, the lymphocytes from the combined BCNU and Bru Pel treatment resulted in mean cpm values intermediate between those detected for the BCNU- and the Bru Pel-treated mice. The mitogenic responses of the splenic B lymphocytes to lipopolysaccharide (LPS) are shown in table 7. Initially, there is no appreciable difference in the incorporation of ^{3}H-TdR between the tumored mice and the untumored controls. However, in tumored controls, there is a steady decrease in the mean cpm after Day 4, and by Day 21 there is a 2.2-fold decrease in mean cpm of the tumored controls relative to the untumored controls. Unlike the lymphocytes from the tumored control mice, lymphocytes from the BCNU-treated group did not steadily decline in mean cpm values. In fact, from Day 4 to 21, they were markedly constant. However, mean cpm values for these BCNU-treated lymphocytes were decidedly lower than the corresponding mean cpm values of the tumored control mice, ranging from a 4.5-fold decrease on Day 4 to a 1.6-fold decrease on Day 21 as compared to the tumored controls. The lymphocytes from the BCNU-treated mice reacted with LPS resulted in a substantial decrease of the incorporation of ^{3}H-TdR in relation to the tumored controls. The reduction in mean cpm ranged from a 9.9-fold decrease on Day 1 to a 1.3-fold decrease on Day 21. The lymphocytes from the Bru Pel-treated mice resulted in mean cpm levels higher than those detected for the tumored control group. The LPS-sensitive lymphocytes from the mice treated with BCNU and Bru Pel, as described above for Con A and PHA, were intermediate in their ability to be stimulated by LPS, being higher than the lympho-

TABLE 5.—Con A proliferative response of splenic lymphocytes from tumor-bearing mice and tumor-bearing mice treated with BCNU and/or Bru Pel

Time (days)	Tumored control (mean cpm)*	Bru Pel alone (mean cpm)	Bru Pel + BCNU (mean cpm)	BCNU alone (mean cpm)	Control (mean cpm)
1	109,559 ± 6140†			13,547 ± 490	117,383 ± 1213
4	100,367 ± 5966			10,439 ± 591	113,635 ± 5007
7	106,855 ± 1910	60,331 ± 2467	13,493 ± 1451	9529 ± 634	103,774 ± 2912
15	96,202 ± 2093	70,101 ± 1765	27,415 ± 2145	13,726 ± 956	100,381 ± 1122
21	96,779 ± 2928	54,753 ± 1588	46,312 ± 2651	12,363 ± 743	105,975 ± 938

*Mean cpm = mean of triplicate cultures mitogen added minus mean of triplicate cultures no mitogen added.
†Mean ± SE of triplicate cultures.

TABLE 6.—PHA proliferative response of splenic lymphocytes from tumor-bearing mice and tumor-bearing mice treated with BCNU and/or Bru Pel

Time (days)	Tumored control (mean cpm)*	Bru Pel alone (mean cpm)	Bru Pel + BCNU (mean cpm)	BCNU alone (mean cpm)	Control (mean cpm)
1	15,010 ± 267†			4852 ± 581	15,596 ± 332
4	17,495 ± 406			4132 ± 411	15,365 ± 1764
7	24,010 ± 2071	35,510 ± 995	8182 ± 466	7961 ± 150	14,158 ± 773
15	14,791 ± 365	23,455 ± 1765	2091 ± 106	− 465 ± 90	16,313 ± 393
21	16,282 ± 2021	19,658 ± 177	3964 ± 152	− 654 ± 18	16,735 ± 1122

*Mean cpm = mean of triplicate cultures mitogen added minus mean of triplicate cultures no mitogen added.
†Mean ± SE of triplicate cultures.

cytes from the BCNU-treated group but lower than the mean cpm values detected for the Bru Pel-alone group.

DISCUSSION

The data presented strongly suggest that Bru Pel stimulates splenic T and B cells of tumor-bearing mice. Stimulation of these T and B cells of the tumor-bearing mice over corresponding values obtained for the non-Bru Pel-treated tumored mice was shown by two different in vitro assays, indirect immunofluorescence and lymphocyte proliferative studies.

The effect of Bru Pel on the T- and B-cell populations was studied in concert with a known cytoreductive agent, BCNU (6,7). We wanted to determine if Bru Pel was effective in the repopulation of splenic T and B cells under tumored conditions. Additionally, BCNU was administered to tumor-bearing mice either alone to reduce T and B cells or in combination with Bru Pel to determine if increases in T and B cells could be attributed to the action of Bru Pel.

Inspection of the immunofluorescent data (tables 3 and 4) reveals that Bru Pel did in fact produce statistically significant increases in the relative percentages of splenic T and B cells over those detected for the untreated tumored controls. BCNU treatment of the tumored mice on the other hand always resulted in significant decreases in T- and B-cell populations. The tumored mice administered BCNU and subsequently injected with Bru Pel always had T- and B-cell percentages intermediate between those of the tumored mice whose T and B cells were stimulated by Bru Pel. These data show that Bru Pel causes a significant increase in the percentages of splenic T and B lymphocytes of tumor-bearing mice relative to untreated tumor controls. Also, these data point out that Bru Pel can partially reverse T- and B-cell depletion caused by the action of a cytoreductive agent, BCNU.

The lymphocyte proliferative studies show essentially the same pattern of stimulatory reactivity of Bru Pel as that detected by immunofluorescence. Bru Pel treatment of tumor-bearing mice resulted in substantial increases of the incorporation of ^{3}H-TdR in PHA-sensitive splenic T lymphocytes (table

TABLE 7.—LPS proliferative response of splenic lymphocytes from tumor-bearing mice and tumor-bearing mice treated with BCNU and/or Bru Pel

Time (days)	Tumor (mean cpm)*	Bru Pel alone (mean cpm)	Bru Pel + BCNU (mean cpm)	BCNU alone (mean cpm)	Control (mean cpm)
1	38,109 ± 2154†			3861 ± 133	40,354 ± 3068
4	44,222 ± 428			9829 ± 399	40,962 ± 2771
7	32,002 ± 746	39,012 ± 375	14,217 ± 942	8664 ± 118	48,057 ± 1404
15	24,870 ± 599	25,869 ± 565	13,018 ± 147	11,431 ± 498	46,997 ± 1527
21	16,691 ± 1149	28,701 ± 706	12,979 ± 502	10,590 ± 743	37,108 ± 781

*Mean cpm = mean of triplicate cultures mitogen added minus mean of triplicate cultures no mitogen added.

†Mean ± SE of triplicate cultures.

6) and LPS-sensitive splenic B lymphocytes (table 7) over corresponding values detected for the untreated tumored mice. Also, Bru Pel treatment partially reversed the decrease in the incorporation of ^{3}H-TdR resulting from the cytoreductive action of BCNU. Only the Con A-sensitive splenic T lymphocytes from Bru Pel-treated tumored mice failed to incorporate more ^{3}H-TdR than the untreated tumored controls (table 5). However, Bru Pel treatment was effective in reversing the effect of T- and B-cell depletion caused by BCNU treatment of the tumored mice. There was a 4.4–6.3-fold increase in the incorporation of ^{3}H-TdR detected for the Bru Pel-treated mice over that detected for the BCNU-treated mice.

In addition to the stimulation of splenic T and B lymphocytes of tumor-bearing mice, Bru Pel treatment resulted in the retardation of tumor size. Tumor size reduction due to the action of Bru Pel was not statistically significant for the Day-7–15 observations. However, tumor size was significantly reduced by Day 21. These observations are in accord with the results of Youngner et al (2) who reported that ip administration of Bru Pel either before or after challenge with Sarcoma 180 tumor cells significantly inhibited the development of ascites tumors.

The observations that Bru Pel has a pronounced stimulatory effect on splenic T and B lymphocytes and has tumor inhibitory properties should make this compound a target for further study. The ability of these T cells to participate in tumor cell killing is presently under investigation in a direct lymphocyte-tumor target cell cytotoxicity assay.

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Immunomodulation With Thiabendazole: A Review of Immunologic Properties and Efficacy in Combined Modality Cancer Therapy [1]

Joel Lundy* and Edmund J. Lovett III [2,3]

SUMMARY

Thiabendazole (TBZ) appears to be an immunorestorative agent, demonstrating maximum immunopotentiation in the immunosuppressed host. Initial in vivo and in vitro immune studies indicate that the drug is most effective when given 24 hours prior to or at the time of administration of antigen. Single doses are more effective than multiple daily doses. One cell population potentiated by TBZ is the macrophage, either by direct activation or secondary to increased lymphokine production. As an adjuvant to conventional cancer treatment modalities, TBZ needs the same host setting as seen with many immunopotentiators. Tumor bulk must be reduced by the primary modality. A dose schedule must be developed in conjunction with each primary modality employed, ie, timing is critical. As an adjuvant in cancer therapy, the drug is most effective when given every other day. Depending upon the primary modality employed, adjuvant TBZ may require dose adjustment.

[Cancer Treat Rep 62:1955–1962, 1978]

Nonspecific immunomodulators can be divided into two general classes: (*a*) those which require a functional immune system, and (*b*) those which appear to have the greatest activity in a setting of suppressed immunity. Particulate biologicals, such as bacillus Calmette-Guérin (BCG) and *Corynebacterium parvum*, are in the first class and are, in general, efficacious when the host is capable of mounting an adequate immune response. Conversely, agents such as levamisole and thymosin tend to restore a level of competence to a suppressed immune system and have variable, if any, effect on the normal immune system. Thiabendazole (TBZ), a low molecular weight (201.26 daltons) synthetic agent, also falls into the second class of immunomodulators.

TBZ is a 2-aryl substituted benzimidazole which has long been used in both medical and veterinary practice as an anthelmintic. Tocco et al (1–3) have investigated the metabolism of TBZ in sheep and man through the use of radiolabeled drug (^{14}C- and ^{35}S-TBZ). In sheep, peak plasma concentrations were reached in 4–7 hours and then decreased rapidly (3). With an oral dose of 1 g of ^{14}C-TBZ in man, peak plasma concentrations of 13–18 μg/ml occurred after 1 hour and rapidly approached 0 between 24 and 48 hours later. Within 48 hours, 92% of the labeled drug had been excreted with 87% in the urine and 5% in the feces (4).

The metabolic fate of TBZ was determined by chromatographic analyses of the urine from sheep that had received an oral dose of ^{14}C- and ^{35}S-TBZ (1). These studies revealed that 98% of the drug was excreted as metabolites and 2% was excreted as pure TBZ. The major metabolite, 5-OH-TBZ, was found as such and as the glucuronide and ethereal sulfate, both of which are enzymatically convertible to the 5-OH form (fig 1).

Toxicity in man has been considered to be of a low magnitude, although cases of liver dysfunction

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and Stevens-Johnson syndrome have been reported.[4]

To evaluate the immunomodulating capabilities of TBZ and to assess its immunotherapeutic efficacy in combined modality cancer therapy, work in this laboratory has been directed to determine: (*a*) the effect of TBZ on immunologic parameters; (*b*) the direct effect of TBZ on tumor cells; (*c*) the effect of the drug, administered alone and in conjunction with conventional cancer therapeutic modalities, on the tumor in vivo; and (*d*) the correlation of observed antitumor effects with in vivo and in vitro alterations of host immunity.

In all of the studies presented in this paper, the tumor used was a 20-methylcholanthrene-induced fibrosarcoma syngeneic to C57BL/6 mice.

IMMUNOMODULATING PROPERTIES OF THIABENDAZOLE

Delayed-Type Hypersensitivity (DTH) Response

The immunogen used in the DTH studies was 2,4-dinitrochlorobenzene (DNCB). This agent was chosen because it is a de novo antigen used to evaluate the DTH response of cancer patients, with apparent prognostic significance (5). A series of experiments was designed to determine the effect of TBZ on the DTH reactivity of normal, tumor-bearing, and immunosuppressed mice. Initially, a single dose of 20 mg/kg of TBZ was administered ip to mice on the day of challenge and paw volumes were determined 48 hours later by a water displacement manometric technique previously described (6). TBZ potentiated normals to a lesser extent (41% increase in response) than tumor-bearers (400% increase in response) (6). This indicated that the drug might be more effective in the immunosuppressed host. Mice given sublethal irradiation and treated with TBZ had almost complete restoration of the DNCB response. Peritoneal exudate cells (a predominant mononuclear cell population) obtained from TBZ-treated mice had the same effect as spleen cells in restoring immune reactivity. Timing of drug administration was critical in this in vivo work. If TBZ was given 24 hours prior to or at the time of administration of antigen, potentiation was seen. However, the drug administered 1 day after antigen or in multiple doses (Days 9–11) was significantly less effective (table 1).

TABLE 1.—Effect of repeat doses of TBZ on the DTH response to DNCB in tumor-bearing mice

Group*	Mean paw vol (μl ± SD)	Statistical significance compared to II† (P value)
I, normal DNCB control	31 ± 8	—
II, tumor DNCB control	18 ± 8	—
III, TBZ on Day 9	68 ± 19	0.001
IV, TBZ on Day 10	64 ± 14	0.0002
V, TBZ on Day 11	32 ± 8	0.04
VI, TBZ on Days 9–11	21 ± 18	0.7

*Mice were sensitized with 250 μg of DNCB on Day 0 at the time of tumor implantation on the low dorsum. TBZ, 20 mg/kg, was administered ip on the days indicated. Mice were challenged with 50 μg of DNCB in the footpad on Day 10. Paw vols were determined on Day 12.

†Statistical significance was determined by the Student's t test.

In a dose-response comparison with levamisole, it was found that both drugs potentiated the DTH response at doses of 2 and 20 mg/kg. In experiment 1 in figure 2, TBZ was more effective than levamisole at 2 mg/kg ($P = 0.01$), while in experiment 2, the reactivity of TBZ-treated mice, at both 2 and 20 mg/kg, was significantly greater than levamisole-treated mice ($P = 0.005$ and $P = 0.02$ respectively). TBZ, at a dose of 100 mg/kg, was not significantly different from controls; levamisole was fatal to mice at 100 mg/kg.

From these data we concluded that: (*a*) TBZ was apparently more effective in immunosuppressed animals; (*b*) the macrophage population is affected, either by direct activation by TBZ or indirectly by increased lymphokine production; (*c*) optimal in vivo timing of the drug is essential to obtain immunopotentiation; and (*d*) TBZ has greater efficacy than levamisole in this system.

Effect of Thiabendazole in a Mixed Leukocyte Culture (MLC)

The MLC was selected for evaluation as a representative immunologic parameter because the reaction of responder leukocytes to allogeneic stimulator cells (irradiated BALB/c spleen cells) may be analogous to the immunologic reaction of the host to neoantigens presented on malignant cells. In fact, recently published studies indicate that tumor-associated or -specific antigens may be altered or derepressed histocompatibility antigens (7).

In a series of in vivo-in vitro experiments, mice were given 20 mg/kg of TBZ ip at 48, 24, 12, or 4 hours before the animals were killed for in vitro testing. While some increase in reactivity was seen in the 4- and 12-hour groups, maximal potentiation

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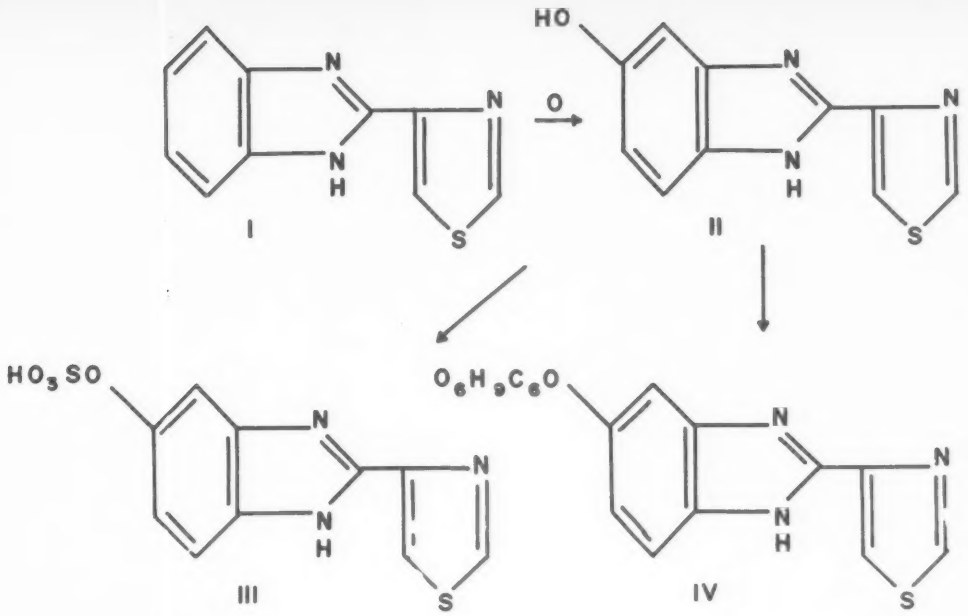

FIGURE 1.—Metabolic fate of TBZ. TBZ (I) is metabolized to 5-OH-TBZ (II), the major metabolite. This form is convertible to the glucuronide (IV) and ethereal sulfate (III), the former by β-glucuronidase and either by gluculase (*from* ref 3).

of the response was seen in the 24-hour group (165% above untreated controls) (fig 3). The group receiving TBZ 48 hours before death was not statistically different from controls (8).

The kinetics of the TBZ effect on the MLC was of note. While the proliferative peak with spleen cells from untreated mice occurred on Day 6 of culture, cells from TBZ-treated animals had the greatest response in the MLC on Day 3 (8). Twomey (9) demonstrated that unlike mitogen-induced blast transformation, where a period of 3 days is needed for brisk mitotic activity, the MLC needed a period of 5–7 days to achieve a mitotic peak. Whether this premitotic period is necessary for antigen processing, mediator production, or some other function leading to a second proliferation signal, it appears to have been eliminated by TBZ treatment.

In addition to causing an alteration in the normal kinetics, the magnitude of the response of TBZ-treated animals was 3.5 times the response of untreated animals, as measured by the area under the circumscribed curves. Either a larger clone of cells was being instructed to respond or the normally reactive clone was undergoing an amplified proliferative response.

Through selective depletion and reconstitution experiments, it was determined that the TBZ-activated cell population was in the plastic adherent fraction and that TBZ-induced potentiation could be transferred to normal lymphocytes by peritoneal cells from TBZ-treated animals. It was concluded that modulation of macrophage activity was possibly responsible for the drug's effect on the MLC (8). Rode and Gordon (10) showed that the neutrophil, the other major cell type in a peritoneal exudate, had no regulatory function in this system. An immune regulatory function has been attibuted to the macrophage in this and several other systems (11).

Direct Reticuloendothelial System Stimulation: Carbon Clearance Assay

In view of the MLC data, it was decided to determine the direct effect of TBZ on the mononuclear phagocytic cell compartment of the reticuloendothelial system. Table 2 shows the accumulated results of three experiments. All drugs were administered 24 hours prior to the assay in the following doses: 20 mg/kg (TBZ and levamisole) and 1.4 mg/mouse (*C. parvum*). The phagocytic index was significantly higher with *C. parvum* than with TBZ (P =

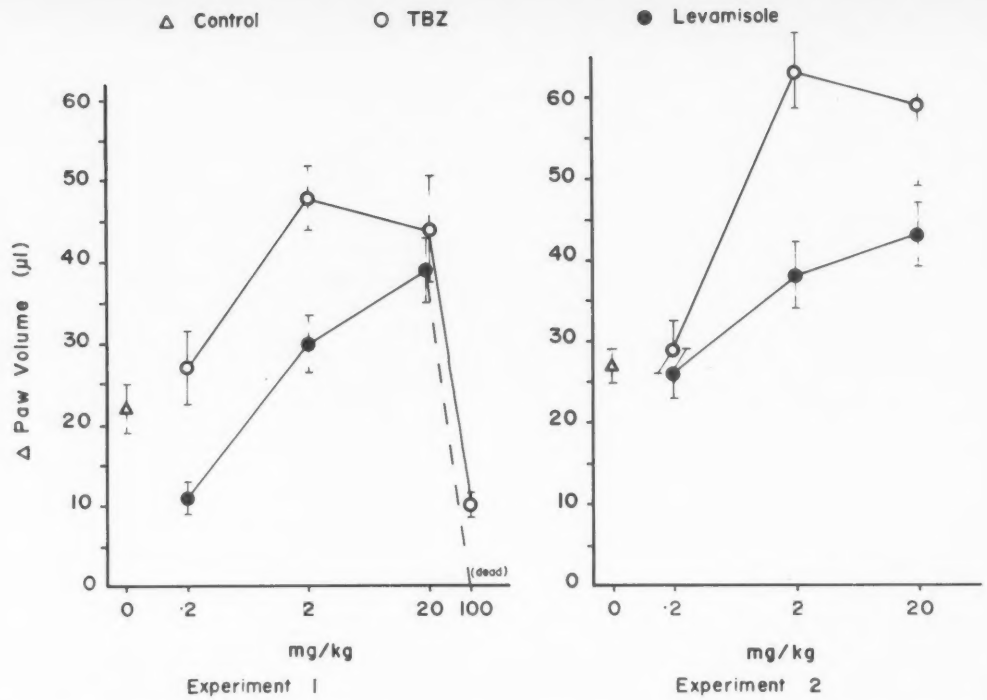

FIGURE 2.—Dose-response comparison of TBZ and levamisole in a DTH response to DNCB in tumor-bearing mice. Drugs were administered ip at the indicated dose at the time of DNCB challenge (Day 10). Paw vols were determined 48 hrs after challenge. Each point = the mean and SD of 8 mice/group in each experiment.

0.01) or with levamisole ($P = 0.04$). While increased carbon clearance was seen in both groups, there was no difference in the phagocytic index of TBZ and levamisole.

These results are similar to those of levamisole described by Hoebeke and Franchi (12). Like the action of TBZ in an MLC, they found that levamisole was most effective in carbon clearance when it was given 24 hours before the assay and was ineffective 48 hours after administration. The data presented here show one significant difference in the activity of the two drugs. While the phagocytic index of both agents is similar, the lack of reticuloendothelial hypertrophy in the TBZ-treated mice resulted in a higher corrected phagocytic index than was obtained with levamisole. The biologic significance of this is unknown.

THIABENDAZOLE IN MULTIMODALITY CANCER THERAPY

Cyclophosphamide and Thiabendazole

Initial studies with TBZ in vitro at a dose range of 10^{-3} to 10^{-12}M indicated that the drug had no cytostatic or cytocidal effect on cultured fibrosarcoma or B16 melanoma cells. TBZ, at a dose of 20 mg/kg, started on the day of tumor transplantation, resulted in a significant delay in tumor growth. However, there was no consistent effect seen on established tumors.

Since the most likely role for immunotherapy seems to be in a setting of minimal residual disease, we evaluated the effectiveness of TBZ as an adjunct to chemotherapy. Following the establishment of a dose-response curve for cyclophosphamide, the agent was administered at an optimal dose of 150 mg/kg on Day 3 following hind-limb tumor cell implantation. This timing was chosen to mimic a clinical situation in which a palpable tumor is present in the host prior to starting chemotherapy. The result was a significant reduction in tumor bulk. TBZ, given at the same time as cyclophosphamide administration or 6 days later, was no better than cyclophosphamide alone in decreasing tumor growth. However, if TBZ therapy was started 4 days after cyclophosphamide, the following results were obtained (fig 4): (*a*) of those mice that responded to cyclophosphamide, 89% responded to

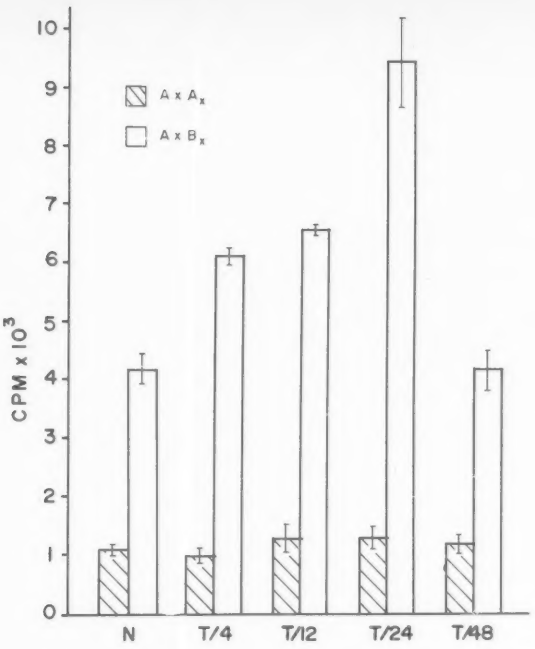

FIGURE 3.—Time to effect of TBZ in an MLC. Responder spleen cells were from untreated C57BL/6 mice or from C57BL/6 mice following administration of 20 mg of TBZ/kg 48, 24, 12, or 4 hrs before they were killed (T/48, T/24, T/12, and T/4 respectively). Stimulator cells received 1200 rads of γ-irradiation. Cultures were terminated on Day 5. Each value = the mean and SD of sample means of 6 replicates in 3 experiments (*from* ref 8).

TBZ, and (*b*) of the cyclophosphamide failures, fewer responded to TBZ, and the duration and magnitude of response was far less ($P = 0.01$). Of importance was the fact that TBZ alone had optimal antitumor activity at a dose of 20 mg/kg, while when used in combination with cyclophosphamide, the most effective dose was 0.2 mg/kg (13) (fig 5). From these experiments, it was evident that the critical factors in successful combined modality therapy with TBZ were: (*a*) reduced tumor bulk, (*b*) alteration of drug dosage dependent upon primary treatment modality employed, and (*c*) optimal timing in relation to the conventional treatment modality employed.

Surgery and Thiabendazole

The 20-methylcholanthrene-induced fibrosarcoma, administered iv (tail vein) as a single-cell suspension, can function as an artificial pulmonary metastasis model. This system is artificial in that the first contact a host has with the tumor is through bloodborne tumor cells. This is in contrast to the natural disease state, in which a primary tumor develops and is subsequently spread, either lymphatically or hematogenously, to various metastatic sites. It does, however, allow study of the role of the host's defense to hematogenously disseminated neoplastic disease without the complicating influence of such factors as blocking factors (either host or tumor in origin) and immunosuppression caused by the primary tumor. In addition, the metastatic insult can be controlled and varied. TBZ started on the day of tumor challenge will protect mice against doses of up to 1×10^4 tumor cells in this system.

Because certain anesthetic agents and a major surgical procedure have been shown by us to result in an increase in artificial metastases and marked impairment of cell-mediated immunity (14), we wanted to determine if TBZ could function as an adjunct to surgery. TBZ given 2 days prior to injection of tumor cells resulted in an increase in pulmonary metastases (unpublished data). However, if TBZ was started on the day of surgery and/or anesthesia and was continued every other day at a dose of 20 mg/kg, the incidence of artificial metastases was reduced to control levels (15) (fig 6). The suppression of cell-mediated cytotoxicity that was seen in surgically treated animals postoperatively was significantly diminished in the TBZ group.

To more closely simulate the human cancer setting, we assessed TBZ as an adjunct to surgery in a

TABLE 2.—Effect of immunopotentiators on a carbon clearance assay

	Treatment (5 mice/group)*			
	Normal	TBZ	Levamisole	*C. parvum*
Body wt	20.133 ± 1.33	19.109 ± 0.801	18.327 ± 0.383	18.176 ± 0.675
Spleen wt	0.620 ± 0.001	0.060 ± 0.001	0.059 ± 0.001	0.100 ± 0.016
Liver wt	0.991 ± 0.035	0.891 ± 0.050	0.946 ± 0.007	1.056 ± 0.050
Body wt/spleen wt + liver wt	19.120 ± 0.784	20.452 ± 0.279	18.235 ± 0.188	15.870 ± 1.092
Phagocytic index	0.013 ± 0.001	0.064 ± 0.001	0.054 ± 0.013	0.078 ± 0.008
Corrected phagocytic index	4.499 ± 0.253	8.102 ± 0.144	6.760 ± 0.481	6.792 ± 0.275

* Drugs were administered to mice ip 24 hrs before the assay was performed: TBZ and levamisole, 20 mg/kg; *C. parvum*, 1.4 mg/mouse.

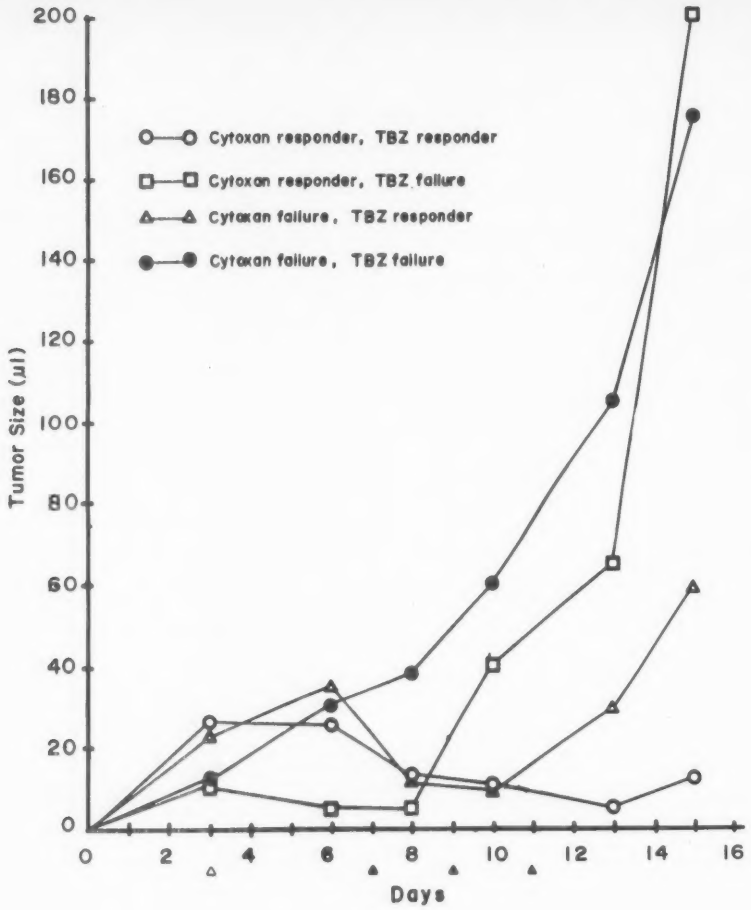

FIGURE 4.—Differential effect of combined chemoimmunotherapy on the growth kinetics of a syngeneic fibrosarcoma in C57BL/6 mice. Mice were injected with 1×10^5 tumor cells in the plantar surface of the left-hind paw on Day 0. Cyclophosphamide (150 mg/kg) was given on Day 3 and TBZ (0.2 mg/kg) was given on Days 7, 9, 11, and 13. Cyclophosphamide response occurred if the tumor size decreased or remained constant from Day 3 to 7. Cyclophosphamide failure was defined as an increase in tumor size from Day 3 to 7. TBZ response meant a decrease in tumor size as compared to the cyclophosphamide-alone control after Day 7, while a TBZ failure indicated no difference in tumor growth as compared to the cyclophosphamide-alone control after Day 7 (*from* ref 13).

spontaneous metastatic system.[5] Again, TBZ given prior to surgery increased the incidence of pulmonary metastases. However, if TBZ (20 mg/kg) was started on the day of anesthesia and amputation of the tumor-bearing limb, there was a significant reduction in the number of pulmonary metastases. This again was associated with the reversal of immunosuppression as determined by cell-mediated cytotoxicity assays in the postoperative period. These experimental data emphasize again the importance of timing of the immunorestorative agent to achieve the desired immunologic or antitumor effects.

[5]Lundy J, Lovett EJ III, Wolinsky S, et al. Immune impairment and metastatic tumor growth: the need for an immunorestorative drug as an adjunct to surgery. Manuscript submitted to *Cancer*.

Restoration of Impaired Mixed Leukocyte Culture Reactivity in Immunosuppressed Mice

Present work in our laboratory is directed to identifying an in vitro immunologic parameter which will best define the optimal temporal relation-

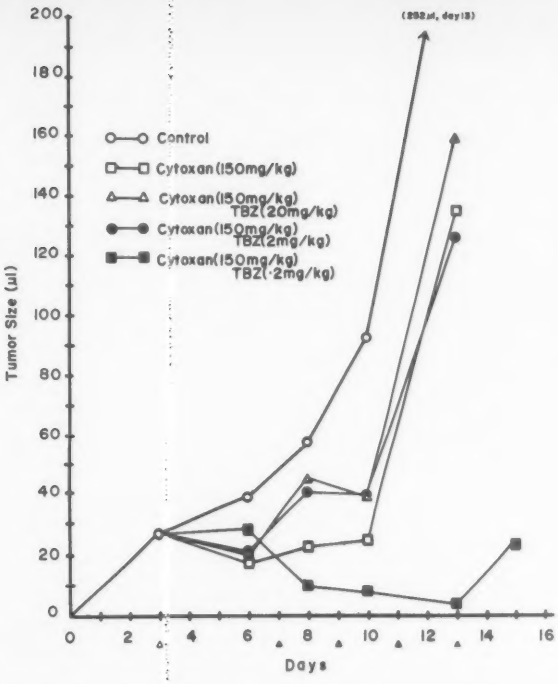

FIGURE 5.—Effect of various doses of TBZ in combination with the optimum dose of cyclophosphamide on the growth kinetics of a syngeneic fibrosarcoma in C57BL/6 mice. Mice were injected with 1×10^5 tumor cells in the plantar surface of the left-hind paw on Day 0. Cyclophosphamide was given on Day 3 and TBZ was given on Days 7, 9, 11, and 13. Tumor size was determined by paw vol measurement. Each point = the mean tumor size of 6 mice (*from* ref 13).

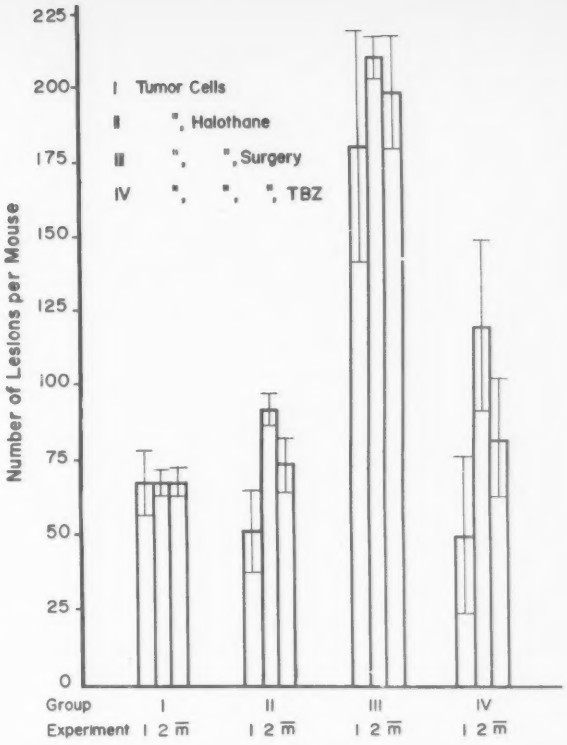

FIGURE 6.—Effect of anesthesia, surgery, and TBZ on the formation of artificial pulmonary metastases. Mice were given tumor cells on Day 0 and killed on Day 12. Pulmonary metastases were enumerated by the method of Wexler. The graph shows 2 experiments and the mean number of metastases (*from* ref 15).

ship between conventional cancer therapeutic modalities and administration of immunotherapy. Initial studies are being carried out in normal non-tumor-bearing mice to alleviate the obvious complicating factors presented by the tumor.

In a group of experiments, normal mice were: (*a*) left untreated, (*b*) treated with an inductive dose of thiopental, (*c*) given both thiopental and halothane anesthesia, or (*d*) surgically treated (hind-limb amputation) under thiopental-halothane anesthesia. A number of animals in each group were treated with TBZ. Subsequently, mice were killed and MLC reactivity was assessed.[6] Immunosuppression was observed in all experimental groups, as compared to controls, 24 and 48 hours following anesthesia and surgery. The most marked suppression was in the combined anesthesia-surgery group at 24 hours (23% of control, $P = 0.0001$). In all experimental groups, treatment with TBZ on Day 0 reversed the suppression demonstrated at 24 hours. Strikingly, the greater the immunosuppression, the more effective the TBZ-induced restoration. Although the immunosuppression persisted, no effect of the Day-0 TBZ administration was seen 48 hours after drug treatment. MLC reactivity of those groups that underwent recovery with TBZ was as suppressed as in their non-TBZ-treated counterparts.

As noted above, we have empirically determined that a second dose of TBZ is needed at this point to minimize the increased number of pulmonary metastases resulting from anesthesia and surgery in the artificial metastatic tumor model. The absence of restored MLC reactivity 48 hours after the Day-0 TBZ administration correlates with the requirements of the optimal dose schedule and indicates that the MLC may serve as a predictive parameter for determining the timing of immunotherapy.

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Isoprinosine: An Overview

Lionel N. Simon* and Alvin J. Glasky [1]

SUMMARY

Isoprinosine appears to have a relatively low degree of both acute and chronic toxicity in both rodent and nonrodent species. Preclinical studies have demonstrated that isoprinosine can inhibit the growth of both DNA and RNA viruses as well as potentiate cell-mediated immune response both in vitro and in vivo. Clinical studies with rhinovirus, herpesvirus, and influenza virus infections in man demonstrated that isoprinosine treatment reduced clinical symptoms and enhanced certain cell-mediated immune responses compared to placebo-treated controls.

[Cancer Treat Rep 62:1963–1969, 1978]

In 1972, isoprinosine [a compound formed by inosine and 2-hydroxypropyldimethylammonium-4-(acetylamino)benzoate, 1:3 molar ratio] (fig 1) was reported to inhibit the growth of a number of RNA (polio, influenza A_2 Hong Kong, and influenza A/PR_8) and DNA (adenovirus and herpesvirus) viruses in tissue cultures by Gordon and Brown (1) and Gordon and Ronsen (2); Chang and Weinstein (3) and Muldoon et al (4) verified and extended these findings.

Subsequent clinical studies indicated that isoprinosine had the ability to enhance several parameters of the immune response while decreasing symptoms in patients either artificially infected with rhinovirus (5) or in natural herpes simplex virus (HSV) infections (6). Hadden and co-workers (7) made the initial observations that isoprinosine, over a broad concentration range, was able to potentiate the phytohemagglutinin (PHA)- and concanavalin A (Con A)-induced lymphocyte proliferation using partially purified human peripheral blood lymphocytes (PBL). Their observations set the stage for the more recent work to be presented in this paper.

This paper presents an overview of isoprinosine's biologic properties and includes a selection of representative examples of the biologic properties of isoprinosine in three main areas: (*a*) preclinical pharmacology, (*b*) preclinical in vitro-in vivo studies bearing on its ability to inhibit viral replication and potentiate the immune response, and (*c*) recent human clinical experiences in viral infectious diseases bearing on its immunorestorative and immunopotentiative (immunomodulatory) properties.

MATERIALS AND METHODS

Materials

Influenza viruses were obtained from ATCC. Prior to in vitro use, a virus pool was prepared in embryonated eggs. For in vivo studies, the virus was passed and titered in mice. The plant mitogen,

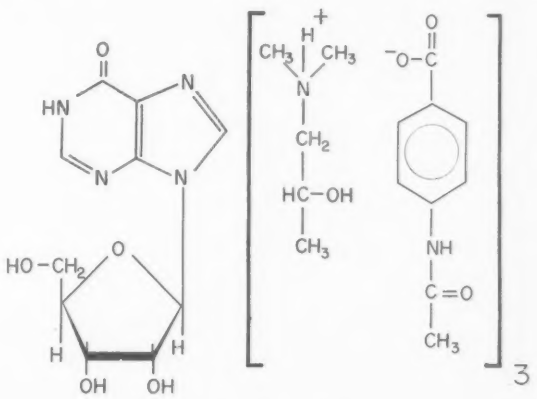

FIGURE 1.—Structure of isoprinosine.

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Con A, was obtained from Calbiochem and PHA-P was obtained from Difco. Tritiated thymidine was obtained from Schwartz-Mann. HeLa and Vero cells were obtained from Gibco. BALB/c mice were obtained from Simonsen Laboratories or Laboratory Supply Co.

Methods

Hemadsorption assay.—The hemadsorption assay was performed using a modified method described by Vogel and Shelokov (8). HeLa- or Vero-cell monolayers were grown in Limbro tissue culture trays using Eagle's minimal essential medium with Earle's balanced salt solution and Hepes buffer, supplemented by adding fetal calf serum (FCS), L-glutamine, chlortetracycline, penicillin, streptomycin, and neomycin.

Spleen cell assay.—The donor mice (12–14 g BALB/c males, 16–18 weeks old) (Laboratory Supply Co) were killed by cervical dislocation. The spleen was removed and placed in a sterile 60-mm Petri dish (Falcon No. 3002) containing serum-free RPMI-1640, supplemented with chlortetracycline (50 μg/ml), Fungizone (2.5 μg/ml), and L-glutamine (2 mmols/ml) (Gibco), and placed on crushed ice. A suspension of spleen cells was prepared in the above media. The suspension was filtered through No. 100 stainless steel mesh (Cellector, Bellco Glass Co). The cell suspension was washed twice and resuspended in 2 ml RPMI-1640, 5% FCS with antibiotics, for each spleen used. The suspension was agitated and the cells were counted in a hemocytometer.

Preclinical Pharmacology

Toxicity studies: acute LD50 determinations.—Male rats, mice, cats, and guinea pigs were treated with a single dose of isoprinosine by either the oral, iv, or ip route. Mortality was observed over a 24–72-hour time period and deaths were recorded. LD50 values were determined using the method of Reed and Munch (9) and the results are shown in table 1. LD50 values for rodents ranged from 7.5 to 10.0 g/kg via the oral route. A similar, low degree of toxicity was observed for other species (5.0 g/kg in the cat) and other routes of administration.

Subacute toxicity.—Isoprinosine was investigated for its subacute toxicity in male and female rats and monkeys during 13 weeks of repeated oral dosing. The tests used dose levels up to 500 mg/kg/day and failed to reveal any significant toxicologic signs or symptoms.

2-Year chronic toxicity: rats.—In a 2-year chronic toxicity test, male and female rats (CD-Sprague-Dawley) were given 375, 750, or 1500 mg/kg/day of isoprinosine in their food. A control group without drug added to the diet was also included. Data obtained on the following parameters revealed no differences between the control animals and animals fed isoprinosine: food consumption, mortality, hematologic profile, clinical blood chemistry, urinalysis, organ weights and ratios, gross and microscopic pathology, and tumor incidence.

TABLE 1.—Acute LD50 toxicity of isoprinosine in 4 species

Route	Species			
	Mouse	Rat	Guinea pig	Cat
Ip	4300	5000	6400	—
Oral	10,000	7500	—	5000
Iv	1850	—	—	—

2-Year chronic toxicity: rhesus monkeys.—In a 2-year chronic toxicity study, rhesus monkeys were given water and 375, 750, or 1500 mg/kg/day of isoprinosine orally. After 12, 18, and 24 months, no abnormalities were found in any of the following parameters: ophthalmology, body weight gains, mortalities and unusual reactions, electrocardiograms, clinical blood chemistry, hematologic profile, urinalysis, and gross and microscopic pathology.

Teratology, fertility, and reproduction.—Pregnant Sprague-Dawley rats were given daily doses of saline or 500 or 1000 mg/kg/day of isoprinosine orally on Days 6–15 of gestation to determine if isoprinosine had any teratogenic effects. No drug effects were found based on the following parameters: maternal body weight, maternal mortality, implantation sites, resorption sites, viable fetuses, fetal external abnormalities, and fetal skeletal and internal development. Rats were also studied to determine if there were any effects on fertility and reproductive performance that could be attributed to isoprinosine. Males were given doses of saline or 500 or 1000 mg/kg/day of isoprinosine orally from age 40 days until the conclusion of mating (60 days later). Females were dosed from 2 weeks (age, 86 days) prior to mating until the termination of the experiment. Isoprinosine administration had no effect on any of the parameters measured (behavioral reactions, implantation sites, corpora lutea, viable fetuses, resorption sites, uterine abnormalities, mating and fertility indices, and the incidence of sires, pregnancy, and parturitions) as well as measures on the number of pups born, ratio of viable pups born, survival rates, body weights, external appearance, and pathologic examination of the weanlings. Teratology studies on albino rabbits were also conducted. Rabbits were treated with saline or 500 and 1000 mg/kg/day of isoprinosine orally on Days 6–18 of

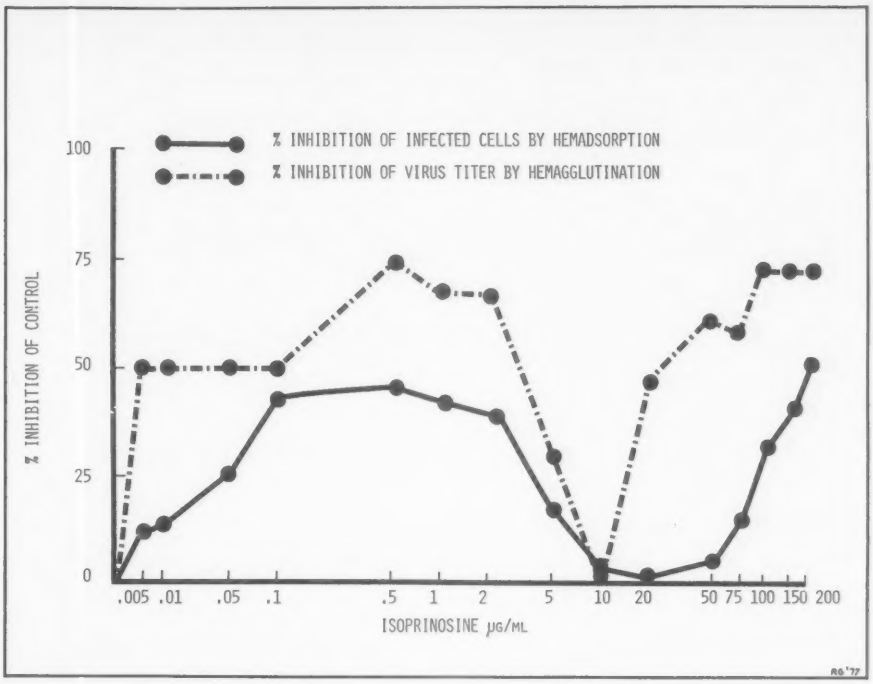
% INHIBITION OF INFECTED CELLS BY HEMADSORPTION
% INHIBITION OF VIRUS TITER BY HEMAGGLUTINATION
% INHIBITION OF CONTROL
100
75
50
25
0
.005 .01 .05 .1 .5 1 2 5 10 20 50 75 100 150 200
ISOPRINOSINE µG/ML

FIGURE 2.—Wide-range dose response of isoprinosine in influenza A/PR$_8$-infected HeLa cells. Effects of isoprinosine on influenza virus growth. In the experiment described, isoprinosine (at the concentrations indicated) was added at the time of infection of HeLa-cell monolayers by influenza A/PR$_8$ virus. The No. of hemadsorbed foci formed 18 hrs after infection was determined in (*a*) placebo-treated (control) and (*b*) isoprinosine-treated (drug) cultures. The % inhibition of control was calculated by dividing the hemadsorbed foci in drug-treated cultures by the hemadsorbed foci in control cultures (solid line). A second measure of viral synthetic activity was taken as the amount of hemagglutinin protein produced intracellularly. Isoprinosine's effect was measured and compared to controls 18 hrs after infection. % inhibition of control was calculated and plotted (dotted line).

gestation. Treatment with isoprinosine was found to have no effects on the measured parameters.

Preclinical In Vitro and In Vivo Studies Relating to Inhibition of Viral Replication and Potentiation of the Immune Response

Because of isoprinosine's metabolic lability and an interest in looking at isoprinosine's effects within early time periods after infection, we examined its effects on influenza virus replication using a modified hemadsorption assay (8). This assay detects changes in cell-surface antigen produced in tissue culture cells after infection with influenza virus at time periods from 2 to 24 hours after viral inoculation. At 18 hours after infection, guinea pig erythrocytes were adsorbed to the infected cell surface producing discrete foci of hemadsorbed cells. These hemadsorbed foci were quantitated using a Bausch and Lomb image analyzer. The data presented in figure 2 show that isoprinosine was able to inhibit the production of hemadsorption foci (HAd foci) by influenza A/PR$_8$ with a biphasic dose-response curve noted over a concentration range of 0.01–200 μg/ml. The inhibition of infected cell formation as judged by a decrease in hemadsorption foci was correlated with a reduction in intracellular viral antigen production as measured using a hemagglutination assay. Similar results have been obtained for 17 different types of influenza viruses including representatives of most subtypes (H_0N_1, H_3N_2, H_2N_2, etc) and type B viruses as well (influenza B/Maryland and influenza B/Massachusetts).

Immunomodulating activity.—In an extension of the earlier observations of Hadden et al (7) on isoprinosine's ability to potentiate plant mitogen-induced human PBL proliferation, we evaluated isoprinosine's ability to augment the Con A- and influenza-induced mouse spleen cell proliferation. Assays were conducted as described in the Mate-

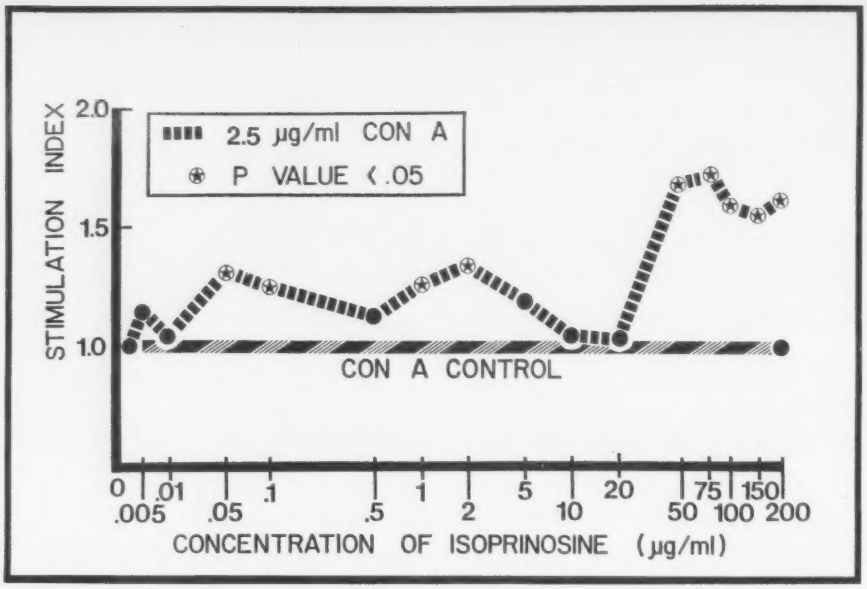
STIMULATION INDEX
2.0
1.5
1.0
2.5 µg/ml CON A
P VALUE < .05
CON A CONTROL
0 .005 .01 .05 .1 .5 1 2 5 10 20 50 75 100 150 200
CONCENTRATION OF ISOPRINOSINE (µg/ml)

FIGURE 3.—Wide-range dose response of isoprinosine with Con A-stimulated murine spleen cells. In the experiment presented, mouse spleen cells were prepared and cultured for 48 hrs in the presence of Con A and the indicated concentration of isoprinosine (µg/ml) (drug) or placebo (control). The effect of isoprinosine on proliferation of mouse spleen cells was calculated by measuring the tritiated thymidine incorporation into DNA in drug-treated cultures (done 3 times) and compared to the placebo-treated controls and expressed as a ratio (stimulation index).

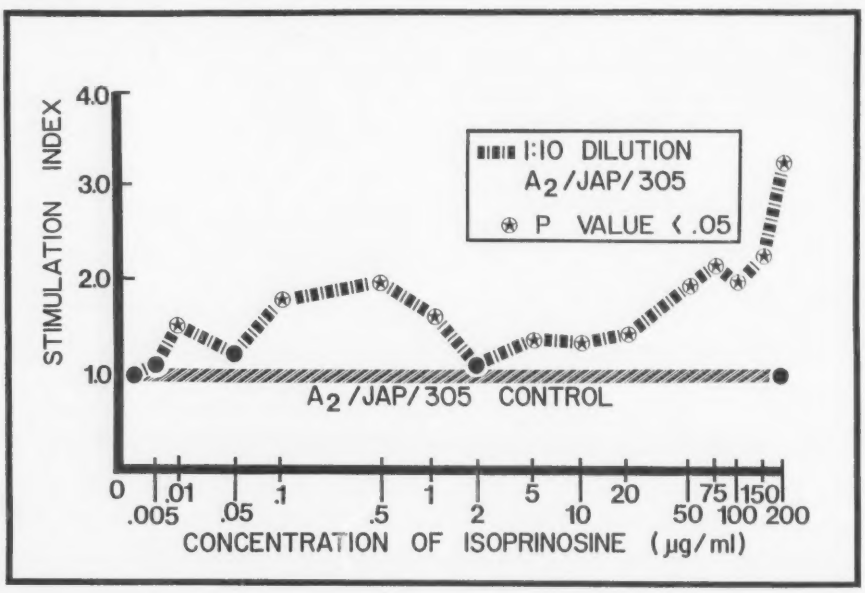

FIGURE 4.—Wide-range dose response of isoprinosine with influenza A_2/JAP/305-stimulated murine spleen cells. In the experiment presented, mouse spleen cells were prepared and cultured for 48 hrs in the presence of A_2/Jap/305 virus and the indicated concentration of isoprinosine (µg/ml) (drug) or placebo (control). The effect of isoprinosine on proliferation of mouse spleen cells was calculated by measuring the tritiated thymidine incorporation into DNA in drug-treated cultures (done 3 times) and compared to the placebo-treated controls and expressed as a ratio (stimulation index).

rials and Methods section and results are expressed as a stimulation index. The stimulation index is a ratio of cpm of ^{3}H-thymidine incorporated into DNA in the presence of drug divided by cpm of ^{3}H-thymidine incorporated into DNA in the absence of drug at varying concentrations of antigen (Con A or influenza). The activity of isoprinosine for Con A and influenza virus A_2/Jap/305 is shown in figures 3 and 4 over a concentration range of 0.01–150 μg/ml. A characteristic double peak or biphasic dose-response curve was noted. Significant stimulation was observed at isoprinosine concentrations as low as 0.05–0.1 μg/ml with a maximum 250% potentiation of the influenza-induced splenocyte proliferation at higher doses. These concentrations of isoprinosine (0.1–0.5 μg/ml) are those observed in human serum after a single oral dose of one 500-mg tablet approximately 1 hour after ingestion. Based on the results from these two studies, we were impressed with the similarity of the biphasic response produced by isoprinosine on inhibition of influenza virus growth (fig 1) and the influenza-induced lymphocyte proliferation (fig 2). As previously reported (10), a correlation coefficient of > 0.90 exists between these two biologic phenomena over both the low concentration range (0.01–10 μg/ml) and the high concentration range (10–150 μg/ml), suggesting perhaps a common biochemical basis for these two biologic activities.

In vivo immunomodulating activity.—Butchko et al (11) have shown that infection (or immunization) of mice with influenza virus to produce a sublethal infection produces alterations in the cellular-mediated immune response of those mice. Within a short time (5–7 days) after inoculation, spleen cells isolated from infected animals have a depressed ability, as compared to noninfected animals, to respond to a nonspecific mitogen-induced lymphocyte proliferation (PHA, Con A). However, their response to the specific infecting influenza virus is enhanced as they recover from the viral challenge. The following experiment was designed to evaluate the interaction in vivo between isoprinosine and the immunomodulating effects of influenza infection. Mice were immunized with influenza virus ip. Drug treatment was begun in one group, while a control group received placebo. Two additional control groups were not immunized; one group received drug and the other received placebo. After 7 days, mice from each group were killed and splenocyte cultures were prepared. Cells from immunized mice not receiving drug showed a suppressed response to Con A stimulation and an elevated response to in vitro stimulation by influenza virus. However, cells from immunized mice that had received isoprinosine in vivo showed a normal Con A response in vitro and a greater than fourfold increase in their specific in vitro response to influenza virus (fig 5). Thus, isoprinosine enhanced the influenza-specific lymphoproliferative response while restoring the suppressed nonspecific response to normal levels. On Day 21 after immunization, the remaining mice were challenged with a lethal dose of influenza virus by aerosol. Mice that were immunized with a high dose of virus (1:5 dilution) were fully protected whether or not they had received the drug. Mice immunized with a low dose of virus (1:100 dilution) were 100% protected if they had also received isoprinosine, but were $< 30\%$ protected with virus immunization alone without drug treatment. In 1976, Renoux et al (12) described the ability of relatively small doses of isoprinosine to enhance a T-cell-dependent B-cell response. Using mice (IOP) injected with sheep rbcs (SRBC) and treated with doses of isoprinosine ranging from 0.005 to 5000 mg/kg, they were able to establish that production of IgM antibodies (Ab) to SRBC was enhanced by a maximum of 225% over the dose range of 0.05–50 mg/kg. The production of IgG Ab was enhanced by a maximum of 198% over a range of 0.5–50 mg/kg. Doses of isoprinosine up to 5000 mg/kg were unable to significantly depress the immune response. Isoprinosine was given as a single ip dose at the time of inoculation with SRBC. Renoux (personal communication) has also reported that Nu/Nu mice treated with 0.5 or 5.0 mg/kg of isoprinosine had an increase in the percent of Th 1,2 (theta) markers.

Clinical Studies

Waldman and Ganguly (5) reported that human volunteers challenged with rhinovirus and treated with either isoprinosine (4.0 g/day) or placebo on a double-blind basis showed a 70% lower mean cumulative symptom score in the isoprinosine group than the placebo group. The PBL isolated from the drug-treated group 8 days after treatment had a normal response to PHA-induced proliferation as compared to the pretreatment value. The PBL isolated from the placebo-treated group after 8 days of treatment showed a 60% decrease in the rate of PHA-induced proliferation.

Wickett and Bradshaw (6), in a double-blind placebo-controlled trial, administered isoprinosine or placebo to a group of 60 patients having either type 1 and/or 2 HSV infections of the oral cavity or genital area. They reported that the isoprinosine-treated group (4.0 g/day total, administered every 4 hours) had a 55% cure rate over a 7-day treatment period compared to a 15% cure rate for the placebo-treated group. The isoprinosine-treated group

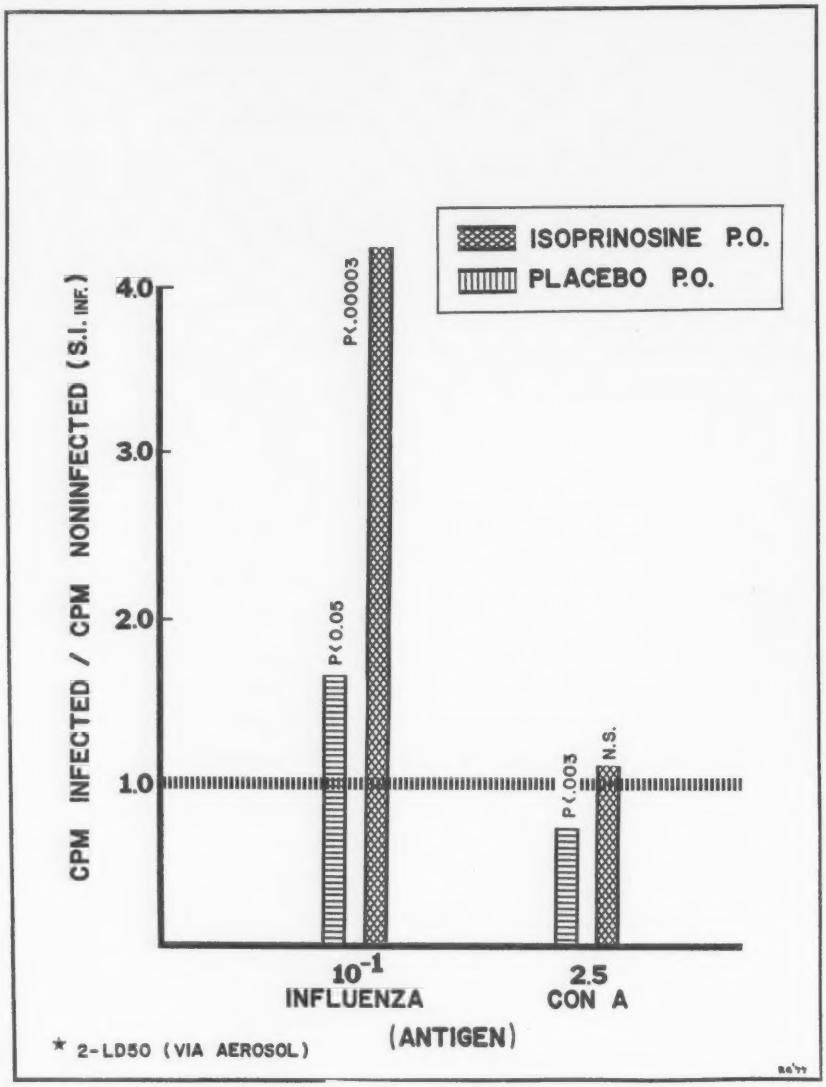

FIGURE 5.—Effect of isoprinosine in vivo on cellular-mediated immune response in flu-infected BALB/c mice. In this experiment, 1 group of mice (BALB/c) were immunized (infected) with influenza A/PR_8 virus. Drug or placebo treatment began at the time of immunization (1% orally in drinking water daily is equivalent to about 600 mg/kg). A second group was not immunized and was treated with either drug or placebo at the time of mock immunization (infection). Spleen cells were isolated from the uninfected and infected animals, stimulated with Con A or influenza A/PR_8 virus, and incubated for 48 hrs. Tritiated thymidine was added after 48 hrs and incubation was carried out for an additional 18 hrs. Incorporation into DNA was measured. The degree of thymidine incorporation into DNA of spleen cells isolated from infected animals was divided by the thymidine incorporated into DNA from noninfected animals and expressed as a ratio (stimulation index) for placebo- or drug-treated animals.

showed a 138% increase in the PHA responsiveness of the PBL after treatment, while the PBL from the placebo-treated group showed a slight decrease or no change. The production of a specific lymphokine, lymphotoxin, was reported to be enhanced 63% after 7 days of treatment with isoprinosine while its production in the placebo-treated group after treatment was depressed 40% (13).

In a double-blind placebo study recently completed, Betz and Douglas (personal communication) showed that in humans challenged with influenza A/Victoria, isoprinosine treatment (*a*) reduced the signs and symptoms of influenza as compared to placebo, (*b*) produced a significant reduction in viral titer in the nasal washings, (*c*) produced an increase in the serum Ab titer compared to placebo treatment, (*d*) produced a significant increase in the influenza-induced lymphocyte proliferation after 3 or 6 days of drug treatment compared to controls, and (*e*) produced a greater increase in the percent of lymphocytes, specifically cytotoxic for influenza-infected cells, as compared to placebo controls. In an earlier study, Waldman et al (14) also reported on a significant reduction in signs and symptoms of influenza by isoprinosine compared to placebo in humans treated with 4.0 g/day and challenged with influenza A/Dunedin strain.

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Isoprinosine Increases the Antitumor Action of Interferon

I. Cerutti,[1,*] C. Chany,[1] and J. F. Schlumberger[2,3]

SUMMARY

The association of relatively low doses of interferon with an immunostimulant (isoprinosine) enhances the antitumor effect of interferon. After combined treatment, the mean survival time, tumor incidence, and final survival rate are significantly increased in mice inoculated with 10^6 Crocker tumor 180/TG cells when compared to mice treated with interferon alone.

[Cancer Treat Rep 62:1971–1974, 1978]

We have previously shown that the antiviral effect of interferon (IF) against encephalomyocarditis virus (EMC) in mice is greatly enhanced by the association of isoprinosine (ISO) (1,2). Indeed, when a single injection of ISO precedes by a few hours an injection of IF (20,000 reference units), 60%–70% of the EMC-infected mice are protected. Under the same experimental conditions, one injection of either IF or ISO alone has no effect.

The inhibitory action of IF on the development of a great variety of tumors in vitro and in vivo is well documented (3–8). It was therefore interesting to investigate the effect of ISO on the antitumor function of IF.

MATERIALS AND METHODS

Drug.—ISO is the *p*-acetamidobenzoic acid salt of *N,N*-dimethylamino-2-propanol:inosine complex, 3:1 molar ratio, and was supplied by Laboratoires Delalande (Courbevoie, France). The white crystalline powder is stable at temperatures up to 150°C, its maximal ultraviolet adsorption rate is at 260 nm, and it is water-soluble (2). The physical and chemical properties have been described elsewhere (9). In our experiments, the drug is used at the concentration of 1 mg/g of mouse weight suspended in phosphate buffered saline.

IF.—Mouse IF is prepared in L929 cells following standard procedures. Newcastle disease virus (F148T strain) is employed to induce IF production. In all experiments, the crude preparation is diluted in Eagle's minimal essential medium (MEM) to a final concentration of 20,000 international reference units/0.5 ml. The specific activity of this preparation is 5×10^4 units/mg of protein.

Tumor cells.—The Sarcoma 180/TG is a mutant of the Crocker tumor 180 resistant to purine metabolic inhibitors (10,11). The tumor is transplanted ip into Swiss mice by inoculation of 10^6 cells/0.5 ml/mouse. The tumor-bearing animal dies within 18–28 days after inoculation.

Animals.—All experiments are performed using Swiss male mice (average weight, 20–25 g) routinely maintained in our laboratory. The cell suspensions, ISO or IF, are injected using the ip route following the chronologic order described later in this paper.

RESULTS

Effect of Combined ISO-IF Treatment on Mean Survival Time (MST)

In previous studies, we have shown that the chronologic sequence of the ISO and IF injections is of great importance (1,2). To obtain an antiviral effect in the mice, the two substances must be administered a few hours apart with ISO always injected prior to interferon. Based on these observations, the experiments are designed as follows. In one group of mice, Crocker tumor 180/TG cells are injected at the same time as ISO and followed 3

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[3]We thank C. Girard for her contribution in the preparation of the manuscript.

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TABLE 1.—Effect of treatment with ISO and IF on MST

Treatment group	No. of injections	No. of animals	Confidence interval (5%)	MST	No. of days animals were observed
ISO + IF	1	20	21.5–34.5	28	60
IF			19.6–33.6	26.5	
ISO			17–26	21	
Controls			17–22	19	
ISO + IF	3	80	22.7–40.3	31.6	100
IF			20–40	30	
ISO			18.5–29.5	24	
Controls			16.5–33.5	25	
ISO + IF	6	60	50–78	64	100
IF			39–69	54	
ISO			29–49	39	
Controls			22–38	30	
ISO + IF	12	50	46–82	64*	100
IF			30–60	45	
ISO			17–29	23	
Controls			18–32	26	

*$P < 0.05$ compared to IF group.

hours later by IF (ISO + IF group). In three parallel control groups, the mice are treated separately with either IF, ISO, or MEM.

The antitumor effect of ISO and IF in combination is analyzed after one, three, six, and 12 injections. In the case of prolonged treatment (three, six, and 12 injections), ISO is administered 3 hours prior to IF three times a week (every 48 hours). After a 60-day observation period using a single injection, no difference is noted between the group treated with ISO + IF and the one treated with IF alone (table 1). However, when these two groups are compared to controls, the MST is slightly higher. During a 100-day observation period, somewhat similar results are obtained with three injections. After six injections, the MST of the ISO + IF treated group is superior to that of the IF group, but both are significantly increased when compared to controls. After 12 injections, the MST of the ISO + IF group is significantly greater than that of the IF group (64 versus 45 days, respectively). In addition, the 5% confidence intervals between the ISO + IF and control groups no longer overlap ($P < 0.05$). Under the same experimental conditions, treatment with ISO alone is ineffective.

Effect of ISO + IF Treatment on the Incidence of Tumors

Treatment with ISO and IF in combination affects significantly the incidence and development of tumors. As shown in figure 1a and b, even after one ISO + IF injection, a slight delay in the incidence of the tumors can be seen. This delay is accentuated with an increasing number of injections and becomes important when the animals are treated with 12 injections for 1 month. Some tumors even regress in the mice that survive for long periods of time. A slight delay in tumor incidence in the ISO-treated mice and a more pronounced delay in the IF group can be observed when compared to controls.

Effect of ISO + IF on the Final Survival Rate

As shown in table 2, ISO + IF treatment enhances the final survival rate of the mice. After only three or six ISO + IF injections, the number of animals surviving for long periods of time is already slightly higher than that of the IF group. With 12 injections, the final survival rate is greatly increased in the ISO + IF group (21 of 50 mice) compared to the IF group (ten of 50 mice) ($P < 0.05$). Only 2% of the animals survive in the ISO and control groups.

DISCUSSION

The data collected here show that ISO enhances significantly the tumor-inhibiting function of IF in mice. The combined ISO + IF treatment rapidly delays the incidence of tumors, especially after 12 injections. In this case, some of the tumors even regress in the animals surviving for long periods of time. The mechanism by which ISO enhances the antitumor action of IF is presently unknown.

It has been shown by Hadden et al (12) that ISO increases the sensitivity of phytohemagglutinin-in-

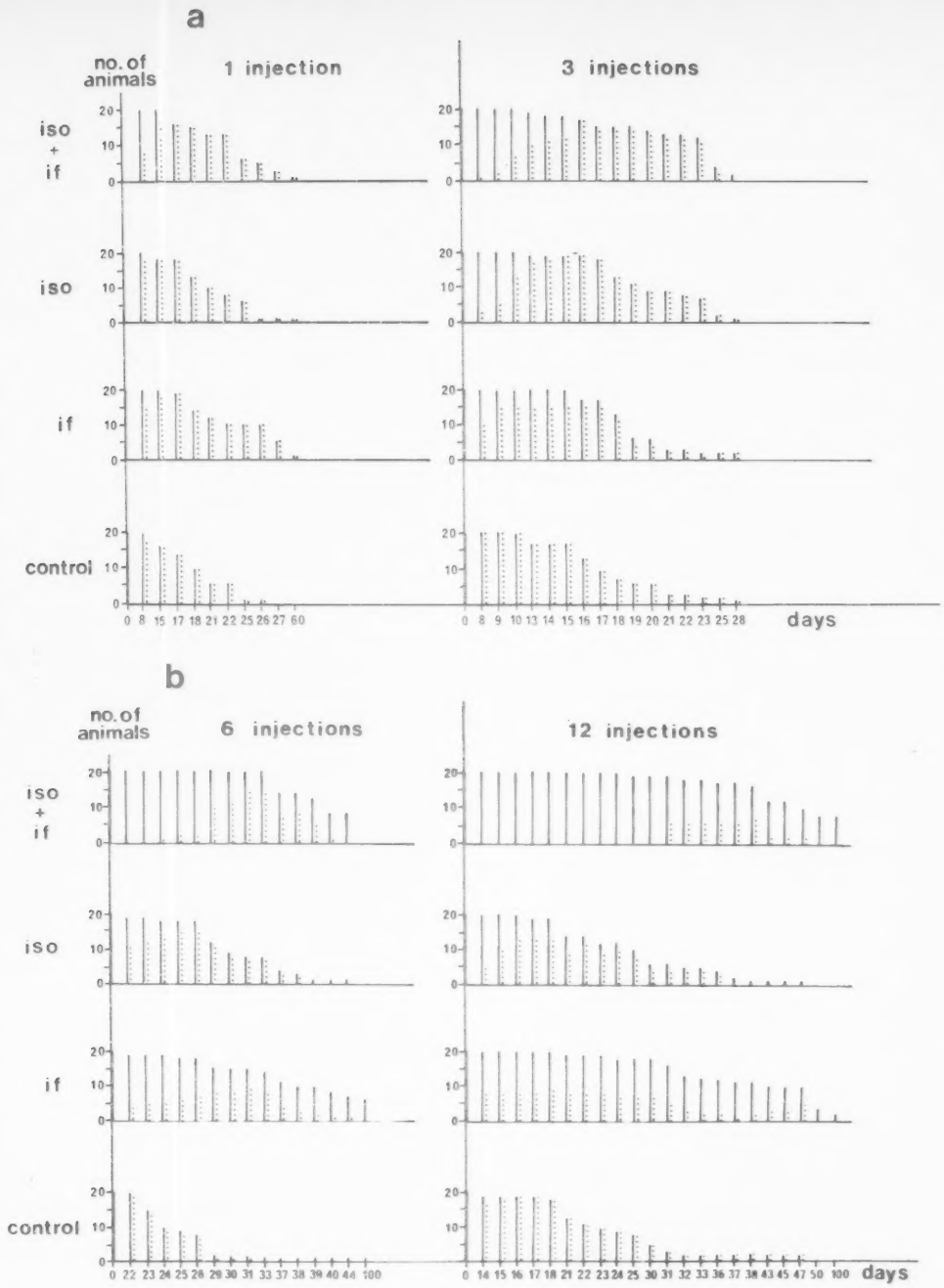

FIGURE 1 (*a* and *b*).—Effect of ISO on the antitumor action of IF. ISO is administered 3 hrs prior to IF (3 times/wk, every 48 hrs). Solid lines = No. of surviving mice; dotted lines = incidence of tumors in these mice.

TABLE 2.—Final survival rate of mice inoculated with Crocker tumor 180/TG cells and treated with ISO and IF administered in combination or alone

No. of injections	Treatment group*			
	ISO + IF	IF	ISO	Controls
1	3/20	3/20	1/20	0/20
3	7/80	5/80	2/80	2/80
6	25/60	18/60	7/60	3/60
12	21/50†	10/50	1/50	1/50

*No. of survivors/No. of mice treated.
†$P < 0.05$ compared to IF group.

duced lymphocyte proliferation. In addition, ISO enhances the suboptimal macrophage-activating factor to induce macrophage and kill *Listeria* (13). ISO augments the helper function of T lymphocytes (14).

The increase of antitumor protection observed when ISO and IF are combined could thus be at least partly related to an increased cell-mediated immunity due to ISO. The role of adjuvants is also supported by a comparable helper effect obtained with formalin-treated *Corynebacterium parvum* associated with IF during Mengo virus infection in mice (unpublished data).

Finally, if the present findings can be reproduced in other animal-cell systems with ISO or other immunostimulants, the therapeutic model described here will be useful in increasing the effects of both IF and adjuvants. Since small amounts of both substances are employed, adverse effects due to an overdose could be avoided.

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Preliminary Characterization in Mice of the Effect of Isoprinosine on the Immune System [1]

Annunciata Vecchi,* Marina Sironi, and Federico Spreafico [2]

SUMMARY

A preliminary characterization of the immunomodulatory activity of isoprinosine in mice was conducted in both normal and tumor-bearing mice. Under the experimental conditions employed, isoprinosine given ip and orally was found to possess some immunomodulatory capacity in various model systems. The compound appeared to preferentially influence T- but not B-cell responses; it did not apparently influence the functional capacity of macrophages, K cells, and NK cells.

[Cancer Treat Rep 62:1975–1979, 1978]

Isoprinosine, the 3:1 molar complex of *N*, *N*-dimethyl-amino-2-propanol *p*-acetamidobenzoate and inosine, has been reported to possess significant antiviral activity in various experimental systems as well as in a number of clinical viral infections, eg, infections sustained by herpes, rhino, and influenza viruses (1,2). The mode of action of this drug is still not completely understood; however, evidence has been presented suggesting that an effect of isoprinosine on host defense mechanisms may at least contribute to its antiviral therapeutic activity (3,4).

In view of the active interest currently focused on immunomodulatory substances, especially those of defined chemical structure and low toxicity, because of their potential in the treatment of a variety of important pathologic conditions, it was of interest to conduct studies aimed at a preliminary characterization of isoprinosine as a nonspecific immunostimulant.

MATERIALS AND METHODS

Animals.—Female $CD2F_1$ and C3H/He mice (weighing 18–22 g at the start of the experiments) obtained from Charles River Italy (Calco, Italy) were used.

Tumors.—The L1210 Cr leukemia, originally provided by Mr. I. Wodinsky (Arthur D. Little, Inc, Cambridge, Mass), and the L1210 Ha leukemia subline, obtained from Dr. E. Mihich (Roswell Park Memorial Institute, Buffalo, NY), were maintained by weekly ip passage in DBA/2 ($H\text{-}2^d$) mice and transplanted in compatible $CD2F_1$ mice; leukemia cell viability was checked microscopically by dye exclusion and was routinely > 95%. For tumor allograft resistance studies, 10^7 L1210 Ha leukemia cells were transplanted ip into histoincompatible C3H ($H\text{-}2^k$) hosts after removal by osmotic lysis of the contaminating erythrocytes from the ascites.

Drugs.—Isoprinosine, obtained from Newport Pharmaceuticals, Inc, Newport Beach, Calif, was freshly dissolved in sterile water and given ip or orally. Adriamycin (AM), a gift from Farmitalia, Milan, Italy, and BCNU, kindly provided by Dr. H. B. Wood, Jr (National Cancer Institute, Bethesda, Md), were freshly dissolved in saline and injected iv or ip respectively.

Lymphocyte stimulation.—Splenocytes (5×10^5) were cultured essentially following the method described by Kirchner et al (5). Graded doses of concanavalin A (ConA) (Calbiochem, San Diego, Calif) and *Escherichia coli* 055:B5 lipopolysaccharide B (LPS) (Difco, Detroit, Mich) were used in each experiment.

Response to SIII.—The optimally immunizing dose of 0.5 μg/mouse of type III pneumococcal polysaccharide (SIII, a gift from Dr. P. Y. Baker, National Institute of Allergy and Infectious Diseases, Bethesda, Md) was administered ip on Day 0

[1]Supported in part by Comunità Economica Europea contract 221-761-BIOI.

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TABLE 1.—Effect of in vivo isoprinosine on the response of mouse splenocytes to ConA*

Drug dose (mg/kg ip × 3)	Day 1		Day 3		Day 5	
	Controls	ConA	Controls	ConA	Controls	ConA
—	4670† (1094)	213,818 (6994)	6793 (2397)	30,593 (327)	8143 (405)	211,432 (5994)
0.05 × 3	4772 (1007)	213,890 (1702)	6445 (1181)	76,844‡ (3076)‡	5841 (635)	245,836 (7175)
0.5 × 3	4716 (1154)	231,580‡ (1650)‡	5596 (143)	51,954‡ (1999)‡	5079 (622)	175,096 (6963)
5.0	4672 (2797)	294,178‡ (6577)‡	2387 (750)	89,383‡ (5199)‡	9288 (392)	180,531 (5530)
50.0	3409 (1266)	254,068‡ (3797)‡	2257 (247)	82,429‡ (1043)‡	4361 (743)	212,316 (6380)

*ConA = 0.4 μg/sample.
†cpm ± (SE).
‡$P = 0.05$ by Dunnet's test.

to C3H mice and the specific anti-SIII plaque-forming cells (PFC) were measured as detailed elsewhere (6).

Clearance of colloidal carbon.—The procedure of Biozzi et al (7) was employed; $CD2F_1$ mice were injected with 160 mg/kg iv of colloidal carbon (commercial India ink, "Pelikan," lot C11/1431/A).

Macrophage cytotoxicity.—The cytotoxic capacity of purified peritoneal macrophages from $CD2F_1$ mice toward SL2 lymphoma cells was evaluated following methods previously described (8).

K-cell activity assay.—To evaluate spleen K-cell activity, chicken erythrocytes were used as targets employing technical conditions previously detailed (9).

NK-cell cytotoxicity assay.—Spleen cells from $CD2F_1$ mice were tested for NK-cell activity strictly following the experimental conditions described by Mantovani et al (10).

Statistical analysis.—Statistical significance was assessed by the Mann-Whitney U test and Fisher's exact test for the survival times and the percent of long-term survivors respectively, and by Dunnet's test for the other results.

RESULTS

A first series of experiments was performed to test whether the effect on mitogen responsiveness reported after the addition of isoprinosine in vitro (3) could also be observed after in vivo administration to rodents. $CD2F_1$ mice were thus given three ip injections of isoprinosine on successive days and the responsiveness of splenocytes was evaluated in vitro at different times thereafter. Table 1 shows that 24 and 72 hours after treatment discontinuation, the responsiveness of lymphoid cells from drug-treated mice to a previously determined optimal concentration of ConA was significantly increased with respect to placebo-injected controls. It may also be seen that although a tendency towards higher responses was seen after the administration of 5 or 50 mg/kg of isoprinosine, this was not always observable; 1 day after treatment the doses of 0.05 or 0.5 mg/kg were just as effective. The effect of isoprinosine under these conditions was only relatively long-lasting since splenocytes tested 5 days after this treatment exhibited normal responsiveness to ConA. In a series of other experiments employing the same conditions, no changes in the response of splenocytes to LPS were detected, thus suggesting that T cells can be a target for the immunostimulatory activity of this drug. This interpretation was supported by the findings obtained when the effects of isoprinosine on the mouse primary response to a T-independent antigen such as the pneumococcal polysaccharide SIII (11) were examined. As presented in table 2, no significant changes in the number of specific anti-SIII antibody-producing cells at peak control response (ie, Day 5 after optimal challenge) were observed after injection of the mice with a dose of isoprinosine capable of increasing the mouse response to sheep erythrocytes, a T-B-dependent antigen (Renoux G, personal communication).

TABLE 2.—Effect of isoprinosine on the primary response of mice to SIII*

Drug dose† (mg/kg ip)	Mean No. of PFC/spleen‡ (range)
—	9300 (7750–11,605)
0.05	6420 (5450–9060)
0.5	9420 (7740–12,260)
5.0	6055 (5100–7190)
50.0	7255 (5455–9650)

*0.5 μg/mouse of SIII ip on Day 0.
†Isoprinosine was given ip on Day 1 and test was performed on Day 5.
‡Results presented are geometric means (in parentheses ± SE).

A limited series of experiments has also been performed to investigate the possible effect of isoprinosine on the response to tumor-associated transplantation antigens. The stimulatory capacity of this drug on a secondary-type responsiveness to

TABLE 3.—Effect of previous treatment with irradiated tumor cells and isoprinosine on the survival of $CD2F_1$ mice challenged with different inocula of L1210 Ha leukemia*

10^6 irradiated cells (Day –8)	Drug (mg/kg) (Day –7 → –1)	No. of L1210 Ha cells (Day 0)	MST†	LTS‡
–	—	10^6	8.8 ± 0.3	0/10
+	—	10^6	19.0 ± 2.7	4/10
+	0.05	10^6	16.0 ± 2.2	4/10
+	0.5	10^6	19.0 ± 3.0	3/10
+	5.0	10^6	16.1 ± 7.7	4/10
+	50.0	10^6	30.0 ± 7.0§	7/10
–	—	10^5	9.9 ± 0.4	0/30
+	—	10^5	21.0 ± 3.9	9/30
+	0.05	10^5	20.4 ± 3.4	14/30
+	0.5	10^5	21.0 ± 2.7	13/30
+	5.0	10^5	20.0 ± 6.0	18/30§
+	50.0	10^5	24.3 ± 4.7	12/30

* Drug treatment and tumor inocula were ip.
† Mean survival time in days ± SE.
‡ Long-term survivors (> 60 days).
§ $P < 0.05$.

tumor cells is shown in table 3. It may be seen that the protection given to $CD2F_1$ mice by a previous immunization with 10^6 X-ray-inactivated (5000 roentgens in vitro) L1210 Ha leukemia cells against subsequent inocula with live cells of the same compatible tumor is significantly increased if the mice are also given a series of isoprinosine injections before leukemia challenge. In fact, statistically significant increases in the percentage of long-term survivors were found in the drug-treated groups (50 mg/kg) when the lower (10^5 tumor cells) inoculum was used, whereas when the challenge was with 10^6 cells, only increases in lifespan were seen. If, on the other hand, treatment with isoprinosine in association with inactivated tumor cells was given after leukemia transplant, no consistent results were observed. In fact, a significant extra increase in survival was seen in only one of three experiments in which $CD2F_1$ mice transplanted sc with 10^4 leukemia cells on Day 0 were inoculated on Day 1 with 10^7 irradiated tumor cells and given single injections of isoprinosine (0.05, 0.5, 5, or 50 mg/kg ip on Day 1). Similarly, when a single or a short sequence of isoprinosine injections was administered to $CD2F_1$ mice bearing L1210 Cr leukemia following chemotherapy with AM or BCNU, no significant therapeutic advantage in terms of increase in survival time over that seen with chemotherapy alone was seen (table 4). It should be noted, however, that for these last types of experiments, a fast growing and very poorly immunogenic tumor such as the L1210 Cr leukemia subline was used, and that under these same conditions other immunostimulants (eg, bacillus Calmette-Guérin) are inconsistently active. Experiments employing other tumor systems as well as a wider range of treatment conditions are already in progress.

TABLE 4.—Effect of isoprinosine and chemotherapy on leukemia-bearing mice*

Isoprinosine (mg/kg ip)	AM (mg/kg iv)	BCNU (mg/kg ip)	MST†
—	—	—	8.3 ± 0.1
—	7.5	—	12.6 ± 0.6
0.5	7.5	—	13.0 ± 0.6
—	10	—	15.4 ± 0.6
0.5	10	—	15.7 ± 0.2
—	—	5	12.2 ± 0.3
0.5	—	5	13.6 ± 0.8
—	—	10	23.6 ± 4.7
0.5	—	10	17.1 ± 1.4
0.5 (× 3)	10	—	14.8 ± 0.6

* 10^5 L1210 Cr leukemia cells were injected ip on Day 0 in $CD2F_1$ mice. AM or BCNU was given on Day 1; isoprinosine was given on Day 5 or Days 2–4.
† Mean survival time in days ± SE.

The influence of treatment with isoprinosine on the growth of an allogeneic tumor was also investigated. Table 5 shows that whereas normal C3H ($H\text{-}2^k$) mice can resist the transplant of high numbers of the incompatible ($H\text{-}2^d$) L1210 Ha leukemia, a high proportion of these animals treated with isoprinosine ip or by the oral route showed progressive tumor takes. The choice of this model system was dictated by our previous studies with another well-known, active chemical immunomodulator, levamisole (LMS). In the experience of many groups, it is

TABLE 5.—Effect of isoprinosine treatment on tumor allograft rejection*

Drug dose† (mg/kg)	Route of administration	MST‡	No. of tumor deaths/total No. of mice
—	—	(11)	1/10
0.05	Ip	8.5 ± 1.3	9/10
0.5	Ip	9.0 ± 1.3	10/10
5.0	Ip	6.8 ± 1.2	8/10
50.0	Ip	7.2 ± 0.6	9/10
0.05	Oral	8.4 ± 0.4	5/10
0.5	Oral	9.2 ± 9.6	4/10
5.0	Oral	8.3 ± 0.3	6/10
50.0	Oral	8.7 ± 0.3	3/10

*10^7 L1210 Ha leukemia cells were injected ip on Day 0 into H-2 incompatible C3H female mice.
†Drug was given from Days 1 to 7.
‡Mean survival time in days ± SE.

often difficult to obtain consistent results in standard models of immune reactivity in animals with LMS, possibly because of its immunorestoring rather than immunostimulatory mode of action. In our hands, however, LMS has consistently shown activity in tumor allograft models through mechanisms which have been only partially clarified (12). It is also worth noting that, in keeping with the reported experience of other groups (13), we have observed the same activity in murine tumor allograft systems for a number of confirmed immunomodulators, including clinically used ones, such as bacillus Calmette-Guérin, *Corynebacterium parvum*, pyran copolymer, NSC-208828, etc.

The data presented above which confirm those data of others obtained in man (3,14) had indicated that T lymphocytes are a target for isoprinosine; thus, it was of interest to examine whether this drug could also affect the functional capacity of other immunocyte subpopulations. No significant differences could, however, be detected between control and drug-treated hosts when the rate of colloidal carbon clearance was measured 1 and 4 days after completion of a 3-day treatment with 0.05–50 mg/kg ip of isoprinosine.

Purified peritoneal macrophages obtained 5 days after administration of a similar treatment did not show a significant nonspecific cytotoxic capacity towards lymphoma cells under conditions where cells collected from *C. parvum*-injected mice were highly active, suggesting that macrophages were not "activated" by the treatments employed. In view of the important role currently attributed to antibody-dependent cellular cytotoxicity and to natural cytotoxicity as defense mechanisms (15,16), the possible effect of isoprinosine on the functional capacity of K and NK cells was also investigated. However, no significant changes with respect to untreated controls were seen in the spleens of $CD2F_1$ mice tested on various days (1–5) after the ip administration of 0.05–50 mg/kg × 3 of isoprinosine and employing YAC lymphoma cells and chicken erythrocytes as targets for NK and K cells.

CONCLUSION

In conclusion, the animal results presented in this paper support the concept that isoprinosine, when administered by the parenteral and oral routes and at doses well-tolerated by mice, has an effect on the functional capacity of the immune system. Our data indicate that T but not B cells can be directly affected by this drug. With regard to other immunocyte subpopulations, our negative results for an activity on macrophages, K cells, and NK cells are to be considered as still preliminary and in need of confirmation using a much wider choice of treatment conditions. In fact, although a number of active treatments were identified, no systematic attempts at recognizing the minimal and optimal drug doses and schedules in the various models have been conducted.

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Effects of Isoprinosine, Levamisole, Muramyl Dipeptide, and SM1213 on Lymphocyte and Macrophage Function In Vitro [1]

John W. Hadden [2,*]

SUMMARY

Four immunopotentiators, levamisole, isoprinosine, muramyl dipeptide, and SM1213, were analyzed in vitro for their effect on phytohemagglutinin-induced lymphocyte proliferation and lymphokine-induced macrophage proliferation and activation to kill *Listeria monocytogenes*. Isoprinosine augmented all three functions. Levamisole augmented lymphocyte proliferation and lymphokine-induced macrophage activation. Muramyl dipeptide and SM1213 induced macrophage activation directly. The effects of these agents on these cells at concentrations relevant to in vivo therapy support their immunopharmacologic application to modify cellular immunity.

[Cancer Treat Rep 62:1981–1985, 1978]

The development of immunopotentiators for use in cancer has led to the discovery of a number of different chemically defined compounds having immunomodulatory properties which have, in turn, been applied to a variety of other disease states, including acute and chronic viral and bacterial infections, immunodeficiencies, and collagen disorders. We have previously characterized this as a "prohost" form of therapy (1) in which the primary therapeutic objectives are the augmentation of cellular immune response and the consequent improvement of a particular disease state. The thymus-derived lymphocyte and the monocyte-derived macrophage are thought to be the principal cellular targets for this type of immunopharmacologic therapy.

In this brief report we compare the actions of four chemically defined compounds by analyzing their effect in vitro on mitogen-induced T-cell-dependent lymphocyte proliferation and lymphokine-induced macrophage proliferation and activation of macrophages to kill *Listeria monocytogenes*. This work has been presented in detail elsewhere (2). The compounds selected for analysis include the following.

Levamisole (LMS).—LMS is a phenylimidothiazole anthelmintic which potentiates immune response (3). LMS has been demonstrated to modify a variety of T-lymphocyte and macrophage responses both in vitro and in vivo and has found clinical application in remission stabilization in cancer, in chronic bacterial and viral infections, and in rheumatoid arthritis (*see* ref 3).

Isoprinosine.—Isoprinosine is a complex of *p*-acetamidobenzoic acid, *N,N*-dimethylamino-2-propanol and inosine (3:1 molar ratio). It has been employed experimentally as an antiviral agent in the treatment of infections with influenza virus, herpesvirus, and rhinovirus. In vitro studies (4) have indicated that this agent is active on lymphocytes and these studies have led to its evaluation as an immunopotentiator (*see* Simon and Glasky in this issue).

Muramyl dipeptide (MDP).—MDP is a substituted monosaccharide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) employed to mimic the immunologic activity of the cell walls of mycobacteria (bacillus Calmette-Guérin [BCG]) (5). The immunomodulatory activities of BCG and its use as a vaccine and in cancer have been extensively reviewed (6). MDP and related water-soluble analogs have shown po-

[1] Supported by Public Health Service grants from the National Institutes of Health, Department of Health, Education, and Welfare, and grants from Newport Pharmaceuticals International, Strategic Medical Research Corporation, The American Heart Association, and the Alton Jones Foundation.

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TABLE 1.—Summary of the effects of immunopotentiators on PHA-induced lymphocyte and MMF-induced macrophage proliferation*

	Lymphocyte proliferation	Optimal concentrations (μg/ml)	Macrophage proliferation	Active concentrations (μg/ml)
LMS	↑ 15%	0.001–0.1	0	—
Isoprinosine	↑ 55%	0.1–100	↑ 35%	1–100
MDP	↓ 10%	0.01–10	↓ 90%	0.01–100
SM1213	0	—	0	—

*Data are expressed as % over control. Compounds were employed over a range of concentrations from 0.001 to 100 μg/ml.

tent immunoadjuvant activity both in vitro and in vivo (5).

SM1213.—SM1213 is a synthetic, substituted monosaccharide developed by Majde and Gordon as an antiviral agent and found to have immunomodulatory activity in vivo (7). This agent is in the early phases of preclinical development and is of particular interest because of its structural relationship to MDP.

These four agents were analyzed in vitro for their biologic effects on relevant response parameters of lymphocytes and macrophages.

MATERIALS AND METHODS

The materials and methods employed in this study have been previously described (2). Ficoll-Hypaque purified human peripheral blood lymphocytes were prepared and phytohemagglutinin (PHA)-induced lymphocyte proliferation was assayed by the incorporation of tritiated thymidine as described (8). Lymphokine (MMF[3])-induced proliferation of guinea pig peritoneal macrophages in monolayer culture was assayed by the incorporation of tritiated thymidine on Days 3 and 5 of culture as described (9,10). Lymphokine (MAF[4])-induced macrophage activation to kill *L. monocytogenes* following 5 days of culture in the presence or absence of MAF was performed during a 6-hour period as described (2). Phagocytosis was quantitated during a 20-minute exposure to *L. monocytogenes* by counting the number of macrophages containing bacteria and the number of bacteria per phagocytic cell on gram-stained monolayers in Labtek chambers. Intracellular killing of bacteria was evaluated by counting the number of cells containing bacteria and the number of bacteria per cell 6 hours after the initial 20-minute exposure.

[3] MMF = macrophage mitogenic factor.
[4] MAF = macrophage activating factor.

RESULTS

PHA-Induced Lymphocyte Proliferation and MMF-Induced Macrophage Proliferation (table 1)

Both LMS and isoprinosine significantly augmented PHA-induced lymphocyte proliferation; neither had a significant effect in the absence of PHA. The maximal effects of isoprinosine were greater compared to those of LMS. The effect of LMS was mimicked by a similar concentration of imidazole. MDP marginally depressed proliferation in the presence of PHA and had no effect in the absence of PHA. SM1213 had no significant effect on lymphocyte proliferation in the presence or absence of PHA. None of the agents had a significant effect on macrophage proliferation in the absence of MMF. In the presence of MMF, isoprinosine stimulated proliferation. Neither LMS nor SM1213 had a significant effect on MMF-induced proliferation. MDP significantly inhibited MMF-induced proliferation. An inactive analog of MDP did not inhibit MMF-induced proliferation.

Activation of Macrophages to Kill *L. monocytogenes* (table 2)

When LMS was added during the 20-minute pulse exposure to the bacteria, it augmented phagocytosis of bacteria by increasing both the percent of cells containing bacteria and the average number of bacteria per cell. During the 6-hour incubation, LMS induced a moderate degree of killing of *Listeria* by the macrophages. If LMS was added at Day 0, little or no effect on phagocytosis and killing was observed on Day 5; however, if LMS was added on Day 0 in the presence of a suboptimal concentration of MAF, it potentiated the effect of MAF to induce macrophages to kill intracellular *Listeria*. If MAF

TABLE 2.—Summary of the effects of immunopotentiators in the presence and absence of MAF on macrophage phagocytosis and activation to kill *L. monocytogenes*

	Phagocytosis	Activation	Active concentration range (μg/ml)
LMS alone	↑	↑	0.03–300
+ suboptimal MAF	0	↑	0.03–300
Isoprinosine alone	± ↑	± ↑	1–100
+ suboptimal MAF	↑	↑	1–100
MDP alone	↑↑	↑↑	0.12–120
+ suboptimal MAF	Not tested	Not tested	—
SM1213 alone	↑↑	↑↑	0.12–12
+ suboptimal MAF	Not tested	Not tested	

was added on Day 0 and LMS on Day 5 during pulse exposure with the bacteria, equivalent data resulted. Thus, LMS added alone or with suboptimal MAF augmented phagocytosis and induced killing of *Listeria*. The results observed with LMS and imidazole were nearly identical.

Isoprinosine had very little effect in the absence of MAF, except that at 1 μg/ml it mildly enhanced killing when added with the bacteria on Day 5. Isoprinosine augmented the effect of suboptimal MAF to induce macrophages to kill *Listeria* whether it was added on Day 0 with the MAF or on Day 5 with the bacteria. Isoprinosine mildly increased phagocytosis when added on Day 5. It significantly augmented the effect of suboptimal MAF to induce macrophage killing.

MDP significantly enhanced both phagocytosis and killing when added on Day 0 of culture or at the onset of the exposure to the bacteria. MDP increased the number of macrophages which ingested bacteria and the number of bacteria ingested per cell. During the 6-hour incubation following the exposure to the bacteria, nearly all of the MDP-treated macrophages completely killed their intracellular bacteria.

SM1213 at concentrations of ≤ 12 μg/ml slightly enhanced phagocytosis and significantly increased killing of intracellular bacteria. SM1213 was active whether added on Day 0 or at the onset of the exposure to the bacteria. Since their effects were quite significant alone, neither MDP nor SM1213 was analyzed in the presence of MAF. None of the four compounds at the concentrations tested had any direct bactericidal or bacteriostatic activity or affected the viability of the macrophage monolayer.

DISCUSSION

These and other data (1,3,4,6) indicate that both LMS and isoprinosine can modulate lymphocyte function. The action of LMS on lymphocyte proliferation in vitro is mimicked by its component imidazole (10) but not by sulfur-containing compounds like dithiosemicarbamate or thiabendazole (3). The effects of both imidazole and LMS on proliferation in vitro are closely paralleled by their ability to increase lymphocyte levels of cyclic GMP (10). The action of LMS in vivo, however, appears to involve the effects of both imidazole and the sulfur moiety (3). Isoprinosine's mechanism of action does not appear to involve early changes in lymphocyte cyclic nucleotide levels and actions linked to RNA metabolism seem likely (12). Both LMS and isoprinosine show double-peaked dose-response curves on PHA-induced lymphocyte proliferation (4,10), suggesting for both compounds that either two-cell populations with differing sensitivities to these compounds are involved in the response or that they have two different types of actions depending on their concentrations. Since PHA-induced lymphocyte proliferation involves both T- and recruited B-cell proliferation (13) and accessory cells are required for the initiation event (14), multiple cellular targets for these drugs is a distinct possibility. The positive effects of LMS and isoprinosine under these circumstances do offer relevant confirmation of cellular targets of their actions for interpreting their effects in vivo on immune response.

MDP and SM1213 did not have significant effects on lymphocyte proliferation. Since MDP has been reported to stimulate lymphocyte proliferation in mouse spleen cells (15), MDP may have differential effects based on species or on the action of accessory cells.

In the assays of macrophage function we examined the effects of the drugs on macrophage proliferation induced by a newly described lymphokine MMF (8,9). MMF in our hands is indistinguishable from MAF, which activates macrophages in vitro to

kill both *L. monocytogenes* and tumor cells (18,19). We employed MAF in the present studies with the presumption that MMF and MAF are the same molecule; however, final biochemical proof of this assumption has not yet been achieved. We can show that the activating factor is not MIF or interferon by exclusion chromatography and isoelectric focusing (unpublished observations). We do not mean to imply that either MIF[5] or interferon is not also an activator of macrophages as has been reported (18–20), since there seem to be a great variety of agents in addition to those presented here which will activate macrophages to be either bactericidal or tumoricidal. The activation process can be achieved within 24 hours with some agents such as lipopolysaccharide, *Listeria* factor, and ascorbate (Englard, Johnson, and Hadden, unpublished data) and by a soluble mediator (21) while 3–5 days is required for activation with MAF (2,18,19). Thus, in the system employed in the present study, both immediate and delayed activation were analyzed and the agents were tested alone, and, if ineffective alone, in combination with a suboptimal preparation of MAF.

LMS had negligible effects on both basal and MMF-induced macrophage proliferation. LMS alone augmented phagocytosis, produced a modest increase in bactericidal capacity, and potentiated the effect of suboptimal MAF to induce bactericidal capacity. Imidazole simulated each of these actions so that the mechanism presumably involves cyclic GMP, as in the lymphocyte (10). Others have demonstrated the effects of LMS to increase phagocytosis and chemotaxis and related these effects to increases in levels of cyclic GMP in phagocytic cells (*see* ref 22 for review). These observations seem particularly relevant to those of in vivo studies in which increased chemotaxis, inflammatory infiltration, or bone marrow recovery in patients or animals treated with LMS has been shown (*see* ref 3).

Isoprinosine's effect on macrophage proliferation was variable depending on the preparation of MMF (2). The positive effects of isoprinosine on MMF-induced macrophage proliferation reported were parallel to those on PHA-induced lymphocyte proliferation and presumably resulted from a similar mechanism. Isoprinosine also augmented phagocytosis and bactericidal capacity, particularly in the presence of suboptimal MAF. The relevance of these in vitro observations with isoprinosine may relate to the in vivo immunoadjuvant actions of isoprinosine seen in mice inoculated with L1210 leukemic cells (*see* Vecchi et al in this issue), influenza virus, and sheep erythrocytes (*see* Simon and Glasky in this issue).

[5]MIF = migration inhibitory factor.

MDP potently inhibited MMF-induced macrophage proliferation with an ID50 of 0.01 μg/ml. Since the drug only slightly inhibited lymphocyte proliferation, a general antiproliferative action can be ruled out. The basis for a selective antiproliferative action on the macrophage remains to be determined. MDP was very active in increasing phagocytosis and bactericidal activity of the macrophage, both early and late. The differential action of MDP to inhibit macrophage proliferation and yet promote activation implies that MDP is a complex agent with different mechanisms of action possibly in separate macrophage subpopulations. In preliminary experiments (ref 23 and unpublished data), we observed that MDP increases both cyclic AMP and cyclic GMP in macrophages. The in vivo relevance of these in vitro observations seems to lie in the potent immunoadjuvant activity of MDP and its active analogs in circumstances where macrophages are known to play a critical role (5).

SM1213, a compound very similar in structure to MDP, was selective in its action in that it activated macrophages and increased their phagocytosis but showed neither proliferative nor antiproliferative activity. Preliminary work indicates that SM1213 increases macrophage levels of cyclic GMP but has no effect on cyclic AMP levels which suggests that SM1213 may share MDP's positive cyclic GMP-related action without its negative, cyclic AMP-related effect on proliferation. Both MDP and SM1213 are 3-substituted monosaccharides and their activity may derive from the effects of low concentrations of sugars to act as hormonal agonists to induce the "glucose effect," also thought to be mediated by cyclic GMP (24). Further experimentation with this agent is needed, both in vitro and in vivo, in order to determine its therapeutic potential.

The in vitro analysis of these four agents has provided a spectrum of observations which, in addition to indicating two target cells of their action and aspects of their mechanisms of action, are provocative to us in predicting their possible applications in vivo. The two agents principally active in facilitating mitogen and lymphokine action, LMS and isoprinosine, have both demonstrated in vivo effects to facilitate on-going immune responses initiated by specific antigenic challenge. The two agents principally active on the macrophage, MDP and SM1213, have been particularly effective in challenge in vivo with infectious agents, the resistance to which is highly dependent on the macrophage. Both types of agents appear to be effective in adjuvant immunization protocols. One wonders what their combined use might yield.

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Immunomodulating Effects of BM 12.531 in Animals and Tolerance in Man

U. Bicker [1,*]

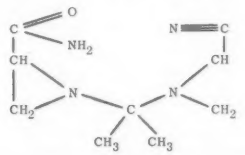
SUMMARY

BM 12.531, a 2-cyansubstituted aziridine, has interesting therapeutic and immunomodulating effects. Antitumor effects in Meth A sarcoma in mice and an increase in resistance to *Candida albicans* infection in mice have been found. It was demonstrated that BM 12.531 can modulate the immune system in animals (DTH, lymphocyte transformation, etc). It was also shown that BM 12.531 can restore the immune response in animals, where this has been suppressed by cyclophosphamide. In a phase I study in cancer patients it was demonstrated that BM 12.531 is very well-tolerated. A dose-dependent increase was found in the percentage of spontaneous T lymphocytes in cancer patients after the administration of BM 12.531.

[Cancer Treat Rep 62:1987–1996, 1978]

We have previously presented the experimental and clinical results with the new immunostimulating compound BM 06.002 (1,2). Recently, we began testing a second compound of this new class of immunomodulating compounds, BM 12.531, because in the first clinical trial with BM 06.002 we have seen that in 10%–20% of the treated patients there was vomiting. However, the first clinical trial of BM 12.531 showed no side effects and we have found that it is very well-tolerated. BM 12.531 also has the advantage that it can be used orally in our experimental investigations.

Chemically, BM 12.531 is the 2-[2-cyanaziridinyl-(1)]-2-[2-carbamoylaziridinyl-(1)]-propane:



The acute toxicity is relatively low (LD50 in rats: ip, 2.6 g/kg, and orally, 3.1 g/kg; LD50 in mice: ip, 2.3 g/kg, and orally, 1.4 g/kg). BM 12.531 has no alkylating or cytotoxic activities either in vitro or in vivo.

In this paper we report on the cancerostatic action of BM 12.531 in the Meth A sarcoma in mice, the potentiation of delayed-type hypersensitivity reaction in mice, the influencing of the primary humoral immune response in the mouse, the potentiation of phagocytosis of macrophages in vitro and in vivo, the influence of the lymphocyte stimulation in vitro, and the reduction of toxicity of cyclophosphamide.

MATERIALS AND METHODS

Compounds.—BM 12.531 (Azimexon) was prepared as described in the patent (3) and administered orally in 0.5% tylose and ip in physiologic saline. Cyclophosphamide (Endoxan, from Asta-Werke AG, Chem. Fabrik, Brackwede, W Germany) and levamisole (Janssen Pharmaceuticals, Beerse, Belgium) were dissolved in physiologic saline. *Corynebacterium parvum* was supplied by Burroughs Wellcome Co (Research Triangle Park, NC), and bacillus Calmette-Guérin (BCG) by the Pasteur Institute (Paris, France).

Animals.—Female NMRI mice (S. Ivanovas GmbH and Co, Kisslegg/Allgäu, W Germany) weighing 16–21 g, female inbred C57BL/6 mice (Zentralinstitut fur Versuchstierzucht, Hannover-Linden, W Germany) weighing 20–25 g, (C57BL/6 × BALB/c)F_1 mice (The Jackson Laboratories, Bar Harbor, Me) weighing 20–25 g, and female and male Sprague-Dawley rats (S. Ivanovas GmbH and Co, Kisslegg/

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Allgäu, W Germany) weighing 150–220 g were used for the experiments. All animals were kept under standardized conditions.

Methodology

Tumor experiments.[2]—The Meth A sarcoma cells (obtained from Dr. H. F. Oettgen, Sloan-Kettering Institute for Cancer Research, New York, NY) were removed from killed animals of the tumor-maintaining strain under sterile conditions. The crushed tumor was mixed with 50 ml of phosphate buffered saline (PBS), pH 7.3, and this was stirred for 7–8 minutes. After centrifugation, the supernatant was drawn off and 40 ml of 0.25% trypsin solution was added to the sediment. This was stirred for 10 minutes, mixed with 50 ml of PBS, and allowed to settle; the supernatant was then discarded. The sediment was subsequently mixed with 40 ml of 0.25% trypsin solution and kept at 37°C for 30 minutes with stirring. Subsequently, it was filtered through sterile gauze, and the filtrate was mixed with 100 ml of Eagle's + H medium. After centrifugation (1000 rpm at 0°C for 10 minutes), the sediment was taken up in 120 ml of medium, the tumor cell count was determined with the aid of trypan blue staining, and the desired tumor cell dilution was prepared with the medium. On Day 0, 1×10^5 cells were injected sc into the necks of female (C57BL/6 × BALB/c)F_1 mice. Treatment with the immunomodulator was started on Day 3 or 7 after the transplantation. All the control animals had died by Day 28. The number of tumor-free animals on Day 28 was determined.

Mackaness delayed-type hypersensitivity reaction.—This procedure, which is a modification of that used by Mackaness et al (4), has been previously described (1).

Influence on primary immune response in mice.—This was determined by the method described by Jerne and Nordin (5).

Tumor growth inhibition assay.—Tumor cells and macrophages were cultured together in a ratio of 1:5 in tissue culture bags as described by Munder et al (6). Each bag (5 × 4 cm) contained 5×10^5 tumor cells (Meth A sarcoma) and 2.5×10^6 macrophages in 5 ml of Dulbecco's modified Eagle's medium (DMEM) + 10% fetal calf serum (FCS). The culture bags were suspended vertically to enable optimal contact between tumor cells and macrophages. After 24, 48, and 72 hours, 1 ml of the cell suspension was withdrawn by puncturing the bags with a 26-gauge needle; the hole closes spontaneously, preventing any contamination. Aliquots (0.2 ml) of the withdrawn cell suspension were put into the wells of microtiter plates (Falcon Plastics, Oxnard, Calif) and pulsed for 3 hours with 0.2 microcurie of ^{3}H-thymidine (specific activity, 21 curies/nm; Amersham and Buchler, Braunschweig, W Germany). The peritoneal macrophages were taken from female (C57BL/6 × BALB/c)F_1 mice pretreated orally with 25 mg/kg of BM 12.531. The macrophages of ten mice per group were used and pooled. For controls, macrophages from mice treated orally with 0.5% tylose were used. The macrophages were obtained 3 or 6 days after administration of BM 12.531.

Phagocytosis of latex particles by peritoneal macrophages of mice.—The macrophages were obtained from the abdominal cavity of untreated female NMRI mice. The animals were killed and immediately 10 ml of DMEM was injected into the abdominal cavity; after light massage, the cell suspension was removed under sterile conditions with a syringe. Enrichment of the macrophages by induced ascites was not carried out since this itself could possibly cause stimulation. BM 12.531, dissolved in FCS, was added to this cell suspension at a concentration of 1 mg/0.5 ml. Pure FCS was added to the controls. Twenty microliters of a latex suspension of normal size (0.81 μm diameter; Difco, Detroit, Mich) was added to the control and experimental suspensions and was incubated at 37°C. After incubating for 1, 10, 20, 30, and 60 minutes, smears were produced and were stained with May-Grünwald-Giemsa stain. One hundred macrophages per smear were counted under the light microscope and the number of phagocytized latex particles was determined. This experiment was repeated three times and the average (± SEM) was plotted graphically.

Lymphocyte stimulation in vitro.—Spleens of female C57BL/6 mice were removed aseptically and teased gently through a 30-gauge mesh in cold PBS. The cell suspension was floated on FCS to remove aggregates. The supernatant containing live and dead cells was withdrawn, again added to FCS, and centrifuged at 500 × g. The pellet was resuspended in DMEM + 10% FCS (Charge M47, mitogen tested; Seromed, Munich, W Germany) and viable cell counts were taken. Spleen cells (5×10^5/well) in 0.2 ml were added to microtiter plates (Falcon Plastics No. 3040, Oxnard, Calif). Various concentrations of BM 12.531 and the mitogen concanavalin A (ConA) (Seromed, Munich, W Germany) were added in 10-μl volumes. The cultures were kept in a humidified incubator aerated with 9% CO_2 in air. After 48 hours, 0.1 microcurie of ^{3}H-thymidine (Amersham and Buchler, Braunschweig, W Germany) was

[2]These experiments were carried out in collaboration with Dr. P. G. Munder, Max-Planck-Institute of Immunobiology, Freiburg, W Germany.

added in 50 μl of DMEM + 10% FCS. The cultures were harvested in a multiple sample harvester (Skatron, Lierbyen, Norway) on glass fiber paper and quadruplicate samples were counted in 3 ml of Rotiscint 11 (Roth, Karlsruhe, W Germany) in a Packard Tricarb Model 3375 (Packard Instruments Co, Downers Grove, Ill).

Induction of leukocytosis.—Groups of ten rats were treated as follows: Group 1, 200 mg/kg of BM 12.531 orally; Group 2, 20 mg/kg of cyclophosphamide iv; Group 3, 200 mg/kg of BM 12.531 orally plus 20 mg/kg of cyclophosphamide iv; and Group 4, 10 ml of 0.5% tylose and 10 ml of NaCl solution/kg iv as control. Blood was collected in a heparinized lance capillary from the retro-orbital venous plexus from fasting animals at 2-day intervals and the leukocytes were counted with a Coulter counter. Groups of ten Sprague-Dawley rats were injected at 7-day intervals with 40 mg/kg of cyclophosphamide. In addition, one group was given 100 mg/kg of BM 12.531 orally at 3-day intervals beginning 3 days before the first cyclophosphamide injection. The control group only was treated with 0.5% of tylose orally and NaCl solution iv alone. Blood was also collected at 2-day intervals and the leukocytes were counted.

Influence of BM 12.531 on the toxicity of cyclophosphamide.—The LD50 of cyclophosphamide was calculated by the method of Litchfield and Wilcoxon (7). Ten mature Sprague-Dawley rats (five female and five male), weighing 150–220 g, were used for each dose group. Doses of cyclophosphamide were 320, 250, 200, 160, or 125 mg/kg. BM 12.531 was given in doses of 10 mg/kg × 3 or 25 mg/kg × 3, 2, 24, and 48 hours after the cyclophosphamide injection. The LD50 was calculated 7 days after the cyclophosphamide administration. The dose-response relationship for the death rate was plotted graphically.

Infection experiments.—Female NMRI mice were injected with 25 mg/kg × 3 doses of cyclophosphamide iv, 1, 3, and 5 days before infection (Group 1). A second group was given, in addition to the cyclophosphamide, doses of 25 mg/kg × 3 of BM 12.531, 2, 4, and 6 days before infection. The animals were infected with *Candida albicans* by iv injection (8).

Clinical investigations.—Initial studies of the tolerance in man have been carried out in cancer patients. These clinical investigations were performed in collaboration with the Faculty of Clinical Medicine of the University of Heidelberg/Mannheim, W Germany (Profs. Stoll, Hoffmeister, Potempa, and Queisser, and Drs. Stosiek and Peters). Patients with different types and stages of cancer were used. BM 12.531 was injected iv. We studied the influence of BM 12.531 on the percentage of activated T lymphocytes in some cancer patients. Control values were determined three times in 13 days in blood obtained from healthy students. The activated lymphocyte count was determined by the method of Wybran and Fudenberg (9).

RESULTS AND DISCUSSION

Results presented in table 1 show that BM 12.531 had a strong antitumor effect in the Meth A sarcoma in mice. The antitumor activity depends on the day on which the treatment was started. A better effect was seen when BM 12.531 (25 mg/kg × 3 doses) was given orally 3 days after tumor inoculation. When the treatment was started 7 days after inoculation of the tumor, the therapeutic effect was not as good because of the high number of tumor cells. In this system, BM 12.531 was better than cyclophosphamide, levamisole, or BCG. *C. parvum* treatment gave results similar to those produced by BM 12.531 treatment. In other tumor systems (Friend virus leukemia in mice, EAC tumor in mice, DS carcinosarcoma in rats, Walker 256 carcinosarcoma in rats) we also found an antitumor effect of BM 12.531 (unpublished data). BM 12.531 is also able to increase the resistance against bacterial and fungal infections (8). BM 12.531 has no direct cytostatic, bacteriostatic, or fungistatic activity. We suggest that the therapeutic effects are caused by the immunostimulating activity of BM 12.531. This compound is a further development of BM 06.002 (1). We began testing this second compound because we found that BM 06.002 caused vomiting in 10%–20% of treated patients.

TABLE 1.—Growth of Meth A sarcoma cells after treatment with various immunomodulators

Immunomodulator	Treatment starting on Day—	
	+ 3 after transplantation* (No. of tumor-free survivors on Day 28/ total)	+ 7 after transplantation* (No. of tumor-free survivors on Day 28/ total)
Control	0/20	0/20
Levamisole (3 × 5 mg/kg) orally	2/10†	1/16†
C. parvum (35 μg/kg) ip	12/20	0/20
BCG (10^7 cells/mouse) ip	5/20	3/20
BM 12.531 (3 × 25 mg/kg) orally	10/20	4/20
Cyclophosphamide (150 mg/kg) orally	0/20	0/20

*10^5 cells/mouse sc in the neck.
†10 and 4 mice respectively died immediately after treatment.

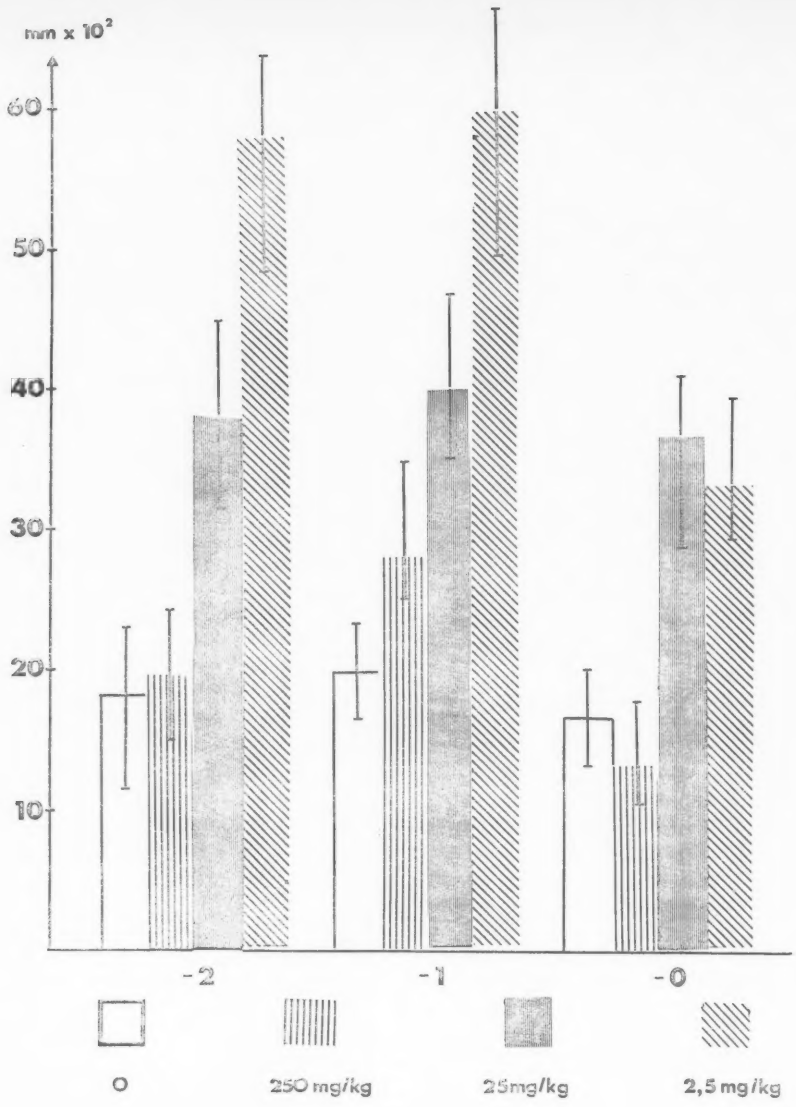

FIGURE 1.—Delayed-type hypersensitivity reaction in female C57BL/6 mice. Animals were immunized 7 days (10^7 sheep rbcs) before challenge (10^8 sheep rbcs). BM 12.531 was administered orally 2, 1, and 0 days before challenge. The difference (mean ± SEM) of the diameter between left and right hind paws is given in mm × 10^2.

Results presented in figure 1 show that BM 12.531 significantly increased the cellular immune response measured by footpad swelling. A significant increase was seen when the compound was given 1 or 2 days before the booster and we found an inverse dose-response relationship. The lowest dose has the best effect. When we carried out these experiments after oral, iv, or ip administration of BM 12.531 we were unable to find any difference. Even the decrease of footpad swelling caused by high doses of sheep rbcs could be prevented when BM 12.531 was given 1 or 2 days before the antigen (10). The influence of BM 12.531 on the delayed-type hypersensitivity has also been described by Schulz et al using the oxazolone method (11).

BM 12.531 also has an effect on the plaque-

TABLE 2.—Antibody formation against sheep rbcs (10^7) after ip application of BM 12.531 in mice (BM 12.531 was given 1 day before the antigen)

BM 12.531 (mg/kg)	Mean No. of PFC/spleen*		
	Day 4	Day 5	Day 6
Control	1514	18,829	1885
100	29,571	36,459	14,714
10	15,914	26,623	11,000
2.5	7714	9085	12,466

*No. of animals = 5.

forming cells in mice. The antibody response to sheep rbcs was stimulated if the antigenic stimulus was low (table 2). On the day of maximum response in the control, the stimulatory effect in the treated animals is diminished and a striking difference is seen on Day 6. BM 12.531 also influences the stimulation of mouse lymphocytes in vitro. Here we found a clear-cut dose-response relationship. A significant increase of the lymphocyte stimulation was measured at doses up to 10 μg/ml whereas there was a decrease at doses > 10 μg/ml (table 3). It is important to note that even high doses of BM 12.531 (> 1000 μg/ml) have no cytotoxic effects as shown by the trypan blue method. It is interesting to note that this dose-response relationship is also seen with BM 12.531 alone without a mitogen. The synergistic effect of BM 12.531 was greatest when low doses of BM 12.531 were added to suboptimal concentrations of the mitogen. The addition of BM 12.531 to ConA-stimulated lymphocyte cultures shows morphologically a dose-dependent decrease in ConA-induced agglutination. The agglutination is partially inhibited or the clusters are much smaller than those in the control culture.

No difference is found when BM 12.531 is given together with the optimal dose of ConA, shortly before the mitogen, or after the mitogen. Therefore, an interaction with the receptors seems to be excluded as ConA binds within minutes. The stimulatory action of phytohemagglutinin works in a similar way (10) so that the special receptor blockade seems unlikely. We also found that the mitogen stimulation and its alteration by BM 12.531 are influenced by the pH. This might indicate that an enzymatic process is involved. The strong dose-response relationship in vitro might therefore offer the possibility of looking for enzymes involved and for studies in cell-free systems.

We also found an activation of macrophages in vitro and in vivo. The results in table 4 show that peritoneal macrophages of BM 12.531-treated mice are able to kill tumor cells in the tumor growth inhibition assay. Using the same tumor cells (Meth A sarcoma) in an in vivo study we were able to demonstrate a therapeutic effect (table 1). We noted

TABLE 3.—Influence of BM 12.531 on spleen lymphocytes of female C57BL/6 mice

Dose of BM 12.531	Mean cpm ± SEM		
	Control	ConA	
		2 μg	8 μg
	BM 12.531 added at the same time as ConA		
Control	910 ± 89	4028 ± 832	54 ± 50
0.1	1176 ± 220	13,466 ± 1694	33 ± 11
1	1246 ± 173	11,858 ± 3346	33 ± 37
10	1321 ± 125	11,173 ± 2589	7 ± 5
50	730 ± 33	5287 ± 1282	2 ± 3
100	288 ± 103	1947 ± 353	10 ± 6
	BM 12.531 given 30 mins before ConA		
Control	796 ± 383	7266 ± 2001	36 ± 30
0.1	1254 ± 28	10,747 ± 2208	59 ± 46
1	1518 ± 63	12,897 ± 2061	25 ± 18
10	1210 ± 173	11,498 ± 941	36 ± 26
50	774 ± 172	5141 ± 943	34 ± 25
100	240 ± 29	2287 ± 814	13 ± 10
	BM 12.531 given 30 mins after ConA		
Control	1224 ± 484	252 ± 226	40 ± 20
0.1	2098 ± 72	10,762 ± 2632	35 ± 42
1	1664 ± 308	15,656 ± 1940	12 ± 16
10	1490 ± 365	13,441 ± 1467	18 ± 17
50	933 ± 78	6825 ± 1765	0 ± 0
100	300 ± 139	1742 ± 535	7 ± 9

TABLE 4.—Tumor growth inhibition by peritoneal macrophages from (C57BL/6 × BALB/c)F_1 mice pretreated orally with 25 mg/kg of BM 12.531*

	% inhibition of ^{3}H-thymidine incorporation of Meth A tumor cells alone		
	Time of incubation		
	24 hrs	48 hrs	72 hrs
Control	38	45	39
On Day −6, 25 mg/kg of BM 12.531	52	79	92
On Day −3, 25 mg/kg of BM 12.531	66	88	94

*pH 7.1; pO_2, 50 torr; 10 mice/group and pooled.

inhibition of the tumor growth when the mice were pretreated 6 or 3 days before obtaining the macrophages. An activation of macrophages in the tumor growth inhibition assay was also found when macrophages and tumor cells were incubated with BM 12.531 directly in vitro (unpublished data). Figure 2 shows also that the phagocytosis of latex particles is increased when mouse macrophages are incubated with BM 12.531, even at a high dose of 1 mg/ml. It is interesting that even this high dose of BM 12.531 stimulates the macrophages in vitro whereas the same high dose inhibits the thymidine incorporation in lymphocytes.

One of the early observations with BM 12.531 was the induction of leukocytosis in rats by BM 12.531 (12). This leukocytosis is dose-dependent and even high doses of BM 12.531 cause a three- to four-fold increase in the number of leukocytes in the peripheral blood in rats. On examination of the differential blood cell counts there is, apart from a slight shift in favor of segmented granulocytes, no other striking change. Other investigators have shown that after administration of BM 12.531, the number of colony-forming units in mice and also the number of

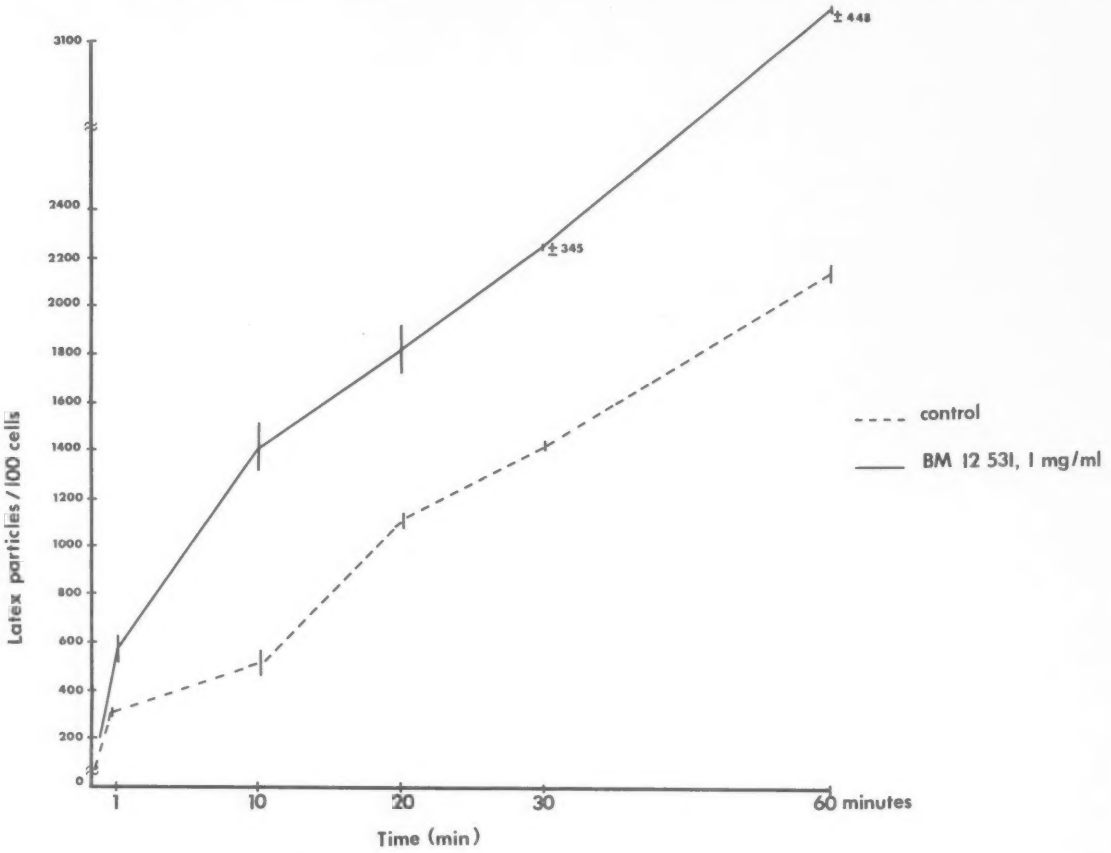

FIGURE 2.—Phagocytosis of latex particles by 100 peritoneal macrophages of mice incubated with BM 12.531. Vertical bars = SEM.

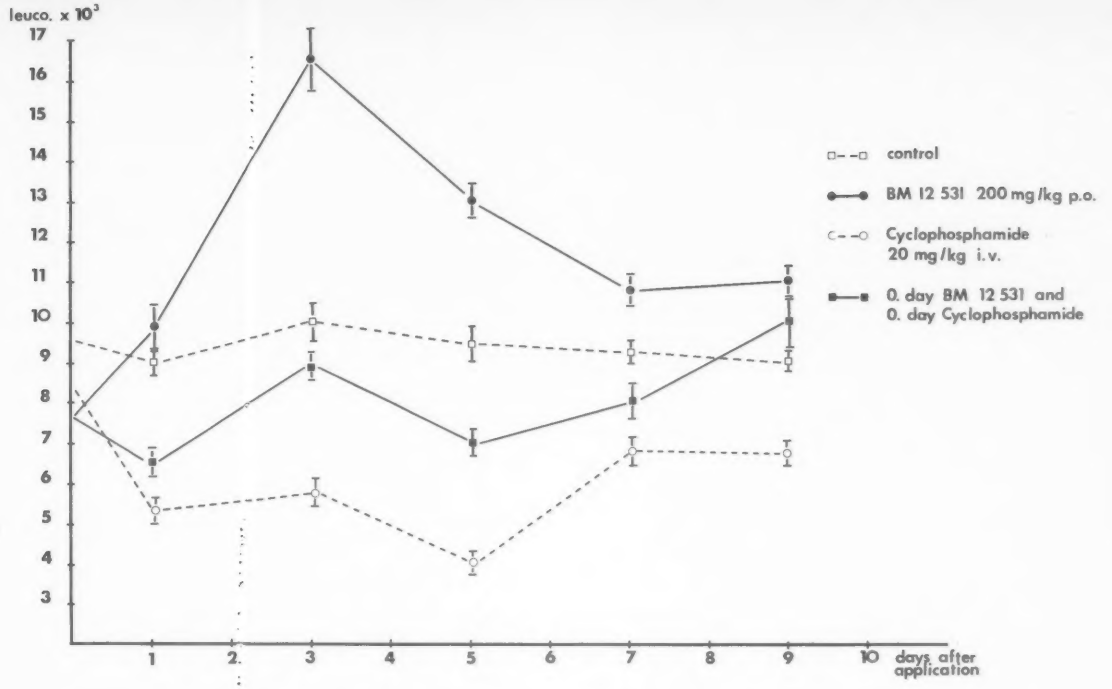

FIGURE 3.—Leukocyte count of rats after administration of BM 12.531 and cyclophosphamide. BM 12.531 and cyclophosphamide were given on Day 0 and blood was collected from the retro-orbital venous plexus at 2-day intervals. Vertical bars = SEM.

nucleated cells in the bone marrow are increased (Reusch et al, unpublished data). Because of these striking effects, we assume that BM 12.531 has bone marrow-stimulating properties.

We know that immunotherapy can only be an adjuvant therapy and therefore we are especially interested in possible interactions with known cytotoxic agents such as cyclophosphamide. Figure 3 shows the effects of (*a*) 20 mg/kg of cyclophosphamide alone, (*b*) cyclophosphamide with 200 mg/kg of BM 12.531, and (*c*) BM 12.531 alone on the leukocyte counts. The leukopenia induced by cyclophosphamide can be positively influenced under these conditions and sometimes can be eliminated entirely. In these experiments, 200 mg/kg of BM 12.531 was given before, simultaneously with, or after the cyclophosphamide and in all cases the leukopenia induced by cyclophosphamide was reduced. This reduction of the leukopenia also was found after repeated administration of cyclophosphamide and BM 12.531 (fig 4).

In order to demonstrate the immunorestorative effect of BM 12.531, we carried out some experiments in immunosuppressed infected mice. Figure 5 shows the survival of mice infected iv with *C. albicans*. If we also administered cyclophosphamide at the doses and schedules shown in figure 5, the animals died within 10 days. The additional administration of BM 12.531 reduces the immunosuppressive effects of cyclophosphamide and the death rate of the treated animals is similar to that of the controls. A similar effect could be seen with other organisms such as *Pseudomonas aeruginosa* (8).

BM 12.531 also influences the acute toxicity of cyclophosphamide. When BM 12.531 was given in doses of 10 mg/kg × 3 or 25 mg/kg × 3 we found a significant change in the LD50 value of cyclophosphamide (fig 6). Schmähl et al (unpublished data) demonstrated in tumor-bearing rats that BM 12.531 reduces the toxicity of cyclophosphamide significantly without abolishing the cytostatic effect of cyclophosphamide.

We believe that BM 12.531 is an interesting new compound because of its therapeutic effects in animal tumor and infection systems and because of its immunomodulating properties. We have completed a 3-month toxicologic study in rats and dogs and the first clinical study with BM 12.531 has been carried out. The tolerance has been investigated in about 80 cancer patients, some of whom have been given

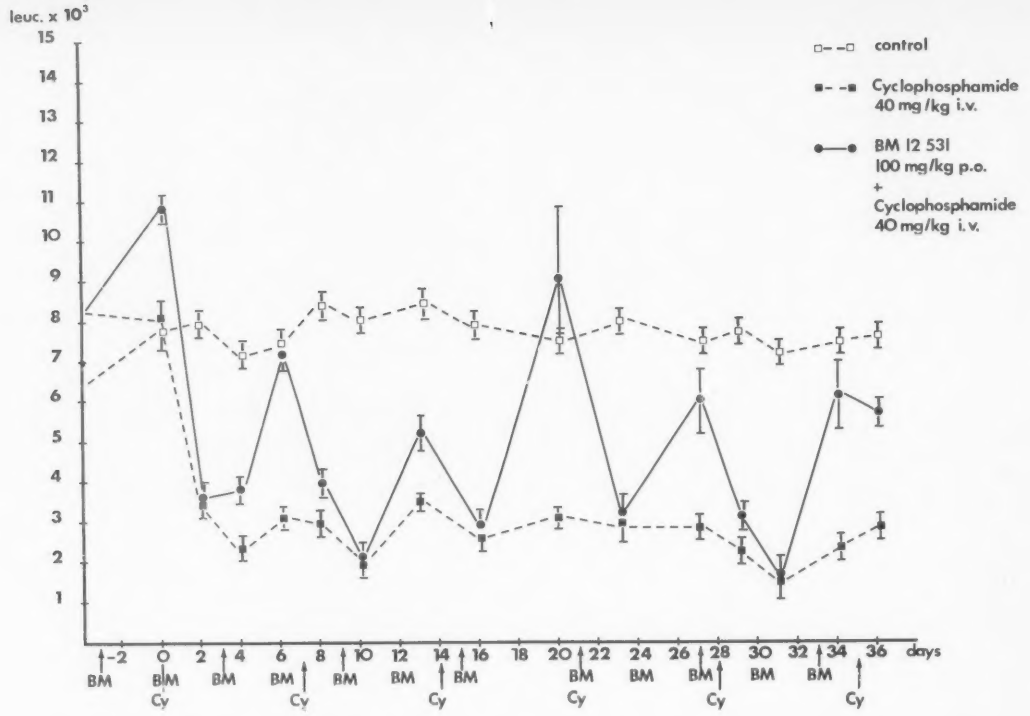

FIGURE 4.—Leukocyte count of rats after oral administration of BM 12.531 and cyclophosphamide. Cyclophosphamide was given at 7-day intervals and BM 12.531 at 3-day intervals. Vertical bars = SEM.

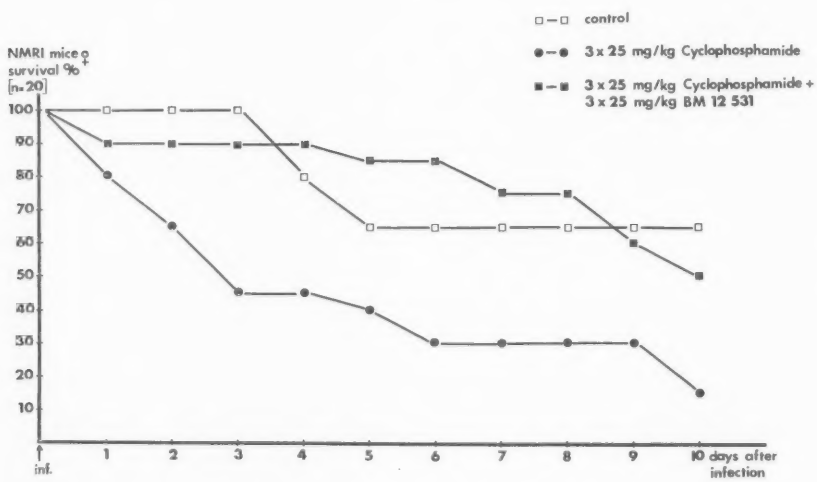
control
3 x 25 mg/kg Cyclophosphamide
3 x 25 mg/kg Cyclophosphamide + 3 x 25 mg/kg BM 12 531
NMRI mice ♀ survival % [n=20]
inf.
10 days after infection

FIGURE 5.—Survival rate of mice infected with *C. albicans* (219) after treatment with cyclophosphamide and/or BM 12.531. Cyclophosphamide was given iv 1, 3, and 5 days before infection and BM 12.531 was administered orally 2, 4, and 6 days before infection. The iv infection dose was 3.1×10^5 organisms/mouse.

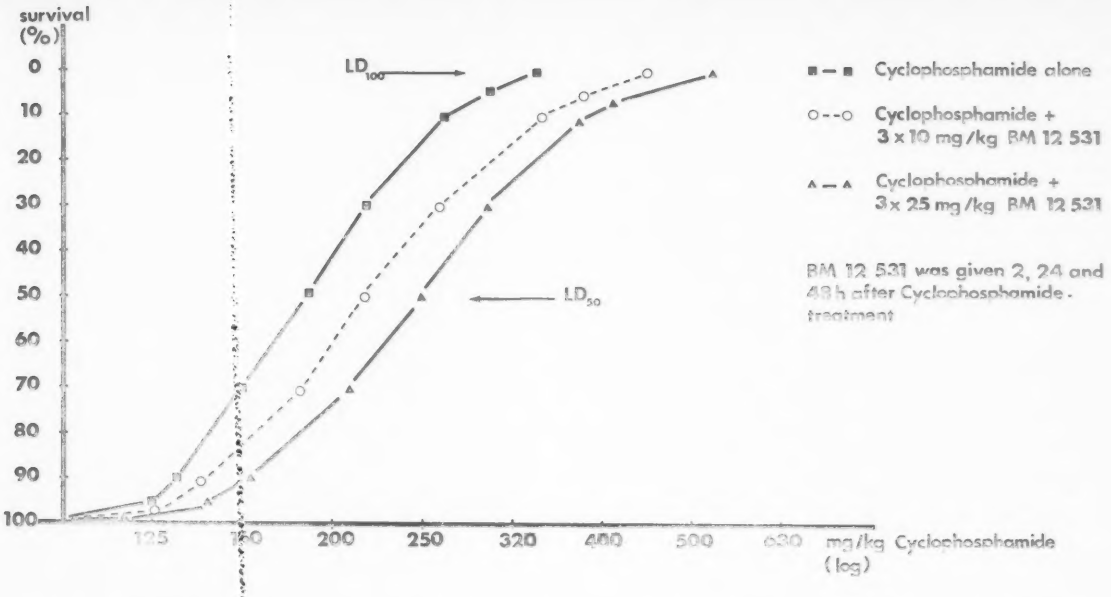

FIGURE 6.—Toxicity dose-response curve of cyclophosphamide alone and cyclophosphamide plus BM 12.531 in rats. BM 12.531 was given orally 2, 4, and 24 hrs after the iv injection of cyclophosphamide and the rate of survival was determined on Day 7 after the cyclophosphamide administration.

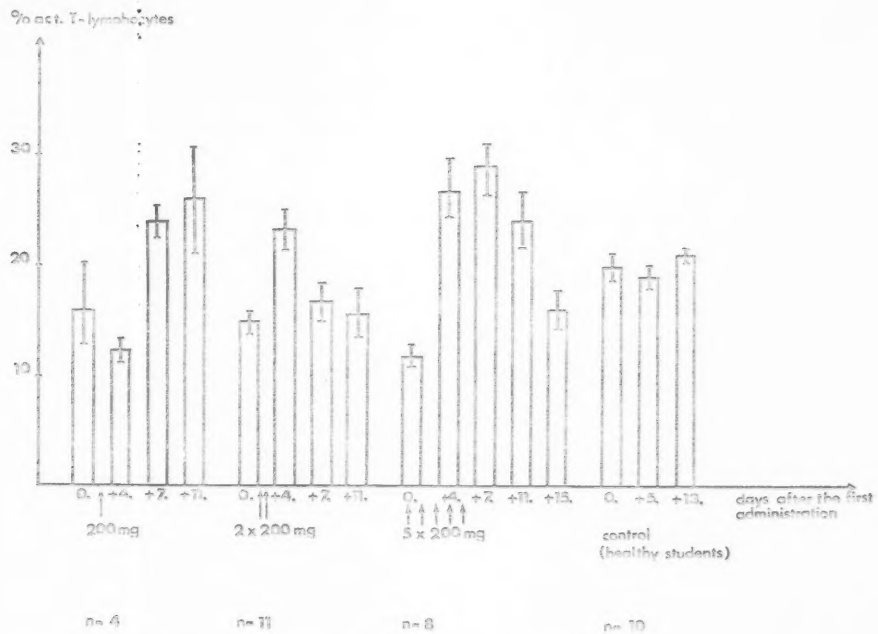

FIGURE 7.—% of activated T lymphocytes in cancer patients after iv injection of BM 12.531. BM 12.531 was given at a dose of 200 mg, 1, 2, or 5 times on consecutive days. Blood was collected from the cubital vein on the days shown in the figure. Vertical bars = SEM.

200 mg on 5 consecutive days with no side effects attributable to BM 12.531. In the higher dose groups we also investigated the influence of BM 12.531 on some immune parameters. We found a dose-dependent influence on the percentage of the activated T lymphocytes, measured by the rosette method of Wybran and Fudenberg (9). A single 200-mg dose and a 200-mg dose × 2 showed the tendency to increase the activated T lymphocytes, and five consecutive doses of 200 mg significantly increased the number of activated T lymphocytes (fig 7).

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ANNOUNCEMENTS

Symposium on "Progress and Perspectives in Lung Cancer Treatment"

Brussels, Belgium, May 3–5, 1979

The European Organization for Research on Treatment of Cancer (EORTC) at the Institut Jules Bordet, Université Libre de Bruxelles, announces a symposium on Progress and Perspectives in Lung Cancer Treatment to be held in Brussels, Belgium, May 3–5, 1979. Participants will be: P. Alberto (Geneva, Switzerland), R. Baldwin (Nottingham, England), N. Bleehen (London, England), M. Cohen (Washington, DC, US), A. De Coster (Brussels, Belgium), A. De Schrijver (Ghent, Belgium), J. Frühling (Brussels), H. Hansen (Copenhagen, Denmark), L. Israel (Paris, France), C. Mountain (Houston, Tex, US), A. Naef (Geneva), P. Rubin (Rochester, NY, US), D. Sanderson (Rochester), and T. Shields (Chicago, Ill, US). The Organizing Committee includes: J. Henry (*Chairman*), R. De Jager, Y. Kenis, J. Klastersky, J. Lustman-Marechal, P. Rocmans, M. Staquet, P. Vanderhoeft, and P. Van Houtte.

Ten topics will be discussed: Early Detection of Lung Cancer; Regional Extension of Lung Cancer; Detection of Distant Metastases; Staging and Prognostic Factors; Limits of Surgical Control; Adjuvant Radiotherapy to Surgery; Immunotherapy and Chemotherapy Adjuvant to Surgery; Treatment of Inoperable Patients by Radiotherapy; Treatment of Inoperable Patients by Chemotherapy; and Small Cell Anaplastic Carcinoma. In addition to invited lectures, free communications will be selected. Abstracts (40 lines maximum) must be submitted by ***February 15, 1979.*** Attendance will be limited to the first 200 registrants; the registration fee is 3000 Belgian francs. ***For further information and submission of abstracts contact:*** Executive Secretary. Ms. D. Eeckhoudt, EORTC Data Center, Institut Jules Bordet, 1 rue Héger-Bordet, 1000 Brussels, Belgium (Phone: 538.57.90-Telex: 22773).

First Meeting of the International Society of Clinical Biostatistics

Brussels, Belgium, May 2 and 3, 1979

The First Meeting of the International Society of Clinical Biostatistics will be held on May 2 and 3, 1979, at the Institut Jules Bordet in Brussels, Belgium. Free communications are invited until ***March 15, 1979.*** The registration fee (including the Society membership for 1 year) is $20.00 or 700 Belgian francs. ***For submission of abstracts and details and information on the meeting please contact:*** Dr. M. Staquet, Institut Jules Bordet, 1 rue Héger-Bordet, 1000 Brussels, Belgium (Phone: 538.57.90-Telex: 22773).

Joint Collaborative Program in Medical Oncology/Hematology

Applications are being accepted from candidates interested in a joint collaborative program in Medical Oncology/Hematology at the University of Massachusetts Medical Center in Worcester. The program lasts for 1–3 years and involves four teaching hospitals with a 1200-bed capacity. It offers intensive clinical and psychosocial experience in Hematology/Oncology, with special emphasis on palliative symptom management, family care, and the dying experience through optional rotations in the Palliative Care and Home Care Programs. Positions are available beginning July 1, 1979.

For information write to: Division of Hematology, University of Massachusetts Medical Center, 55 Lake Ave No, Worcester, Mass 01605.

Seminars in Oncology at Roswell Park Memorial Institute

Buffalo, New York

There will be a seminar on "Re-evaluation of Multimodal Treatment of Melanoma" on March 8, 1979, organized by the Department of General Surgery at Roswell Park Memorial Institute. The chairperson is Dr. E. Douglas Holyoke.

There will be a $25.00 registration fee. ***For further information contact:*** Ms. Claudia Lee, Cancer Control, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.

Postgraduate Course in "Advances in Hematology and Oncology"

New York, New York, April 23–26, 1979

The New York Hospital-Cornell Medical Center, Department of Medicine, Division of Hematology-Oncology, is offering a Post Graduate Course in "Advances in Hematology and Oncology" on April 23–26, 1979. The objective of this course is to review and update the field of Hematology-Oncology including recent advances in diagnosis, treatment, and pathophysiology. Subjects will be covered in sufficient depth and scope to serve as an adequate review for physicians preparing for subspecialty certification. In addition, the course will serve as a stimulating update for knowledgeable internists and practitioners. The course has been awarded 28 hours of Category 1 continuing medical education credits by the American Medical Association.

Inquiries should be addressed to: **Dr. Ralph L. Nachman, Professor of Medicine, Chief, Division of Hematology, The New York Hospital-Cornell Medical Center, Rm F-544, 525 E 68th St, New York, NY 10021.**

Fourth Annual "Symposium on Advances in Cancer Treatment Research"

Baltimore, Maryland, April 19–21, 1979

The Baltimore Cancer Research Center, National Cancer Institute, and the Program of Continuing Education of the University of Maryland School of Medicine are pleased to announce their 4th Annual "Symposium on Advances in Cancer Treatment Research," to be conducted in Baltimore, Maryland, April 19–21, 1979, at the Friendship International Hotel.

An experienced and internationally recognized faculty will participate. This Symposium is designed to update physicians and surgeons on some of the most recent developments in cancer treatment and current research including areas of clinical management, etiology, and cellular biology. Faculty members will be available throughout the Symposium to discuss any questions or interests of individual course registrants. A visit to the Baltimore Cancer Research Center can be scheduled for interested persons. The course is accredited for 16 hours of Category I credit for the PRA of the AMA by the Program of Continuing Education, University of Maryland School of Medicine.

For further information please contact: **Jack Mason, PhD, Assistant Dean for Continuing Medical Education, 10 S Pine St, Rm 300E, Baltimore, Md 21201 (phone: 301-528-3956).**

14th Annual San Francisco Cancer Symposium on "Body Image, Self-Esteem and Sexuality in Cancer Patients"

Hyatt on Union Square, San Francisco, California, March 23–24, 1979

The West Coast Cancer Foundation, in association with the Northern California Cancer Program and St. Mary's Hospital and Medical Center, presents "Body Image, Self-Esteem and Sexuality in Cancer Patients," a symposium for physicians, nurses, psychologists, counselors, social workers, and other health professionals.

The registration fee is $125 for physicians (with CMA credit), $50 for non-M.D. health professionals (with credit), $35 for non-M.D. health professionals (without credit), and $10 for residents and students. ***For further information contact:*** **Jerome M. Vaeth, MD, West Coast Cancer Foundation, 50 Francisco St, Suite 200, San Francisco, Calif 94133 [(415) 981-4590].**

CORRECTIONS

The authors of the article "Combination Chemotherapy for Ovarian Carcinoma With Cyclophosphamide, Adriamycin, and *cis*-Dichlorodiammineplatinum(II) After Failure of Initial Chemotherapy" which appeared in the July 1978 issue of *Cancer Treatment Reports* (page 1021) regret that they inadvertently omitted Dr. Gunther Deppe's name from the list of authors for this paper. His name should have been included as the sixth author.

The authors of the article "Estramustine Phosphate in the Treatment of Advanced Malignant Melanoma" which appeared in the September 1978 issue of *Cancer Treatment Reports* (page 1329) regret that they incorrectly reported the dosage of estramustine phosphate received by their group of patients. The correct dosage given was 15 mg/kg/day, not 15 mg/m^2 as reported.

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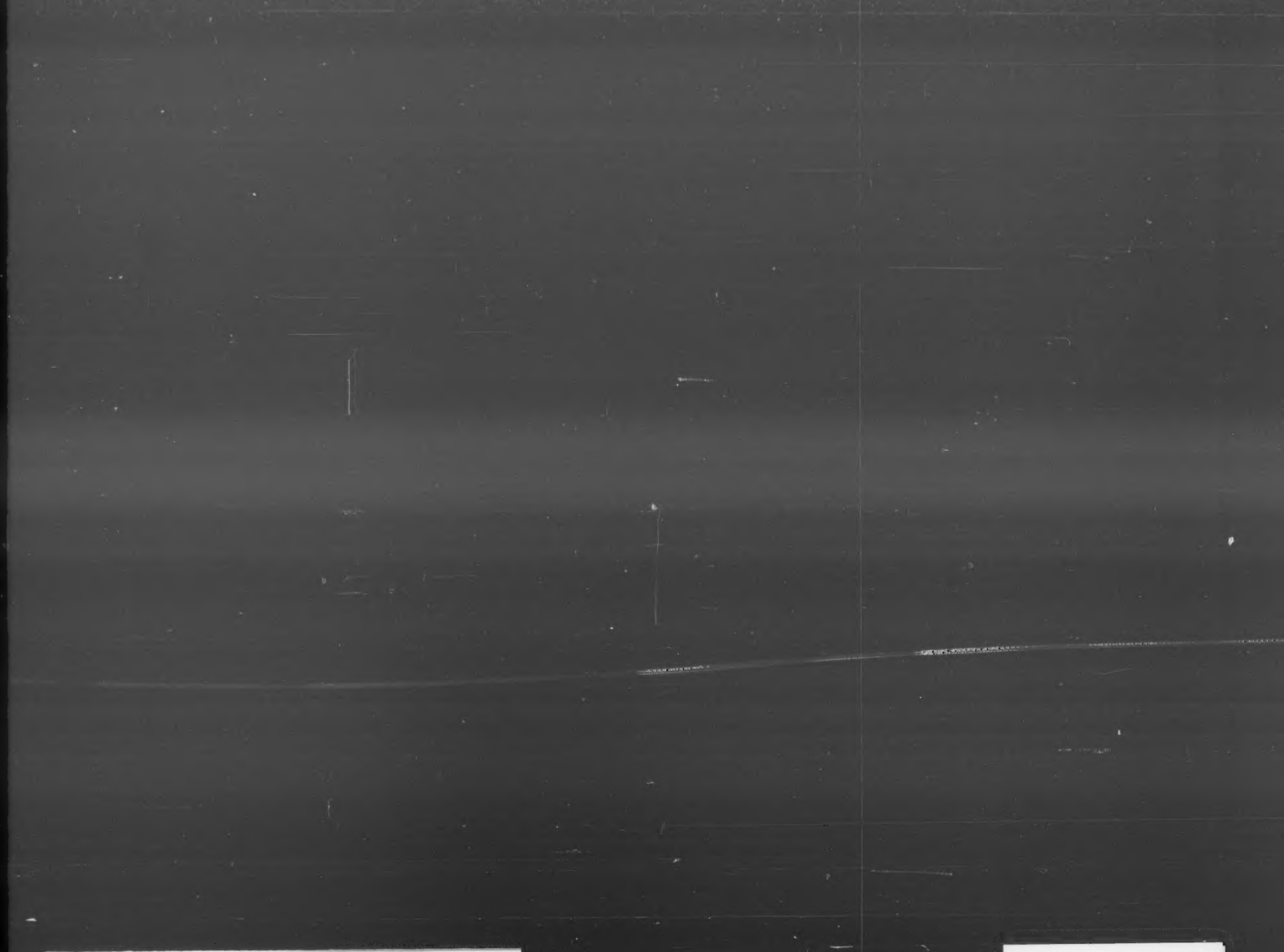
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